

**Identification of Chemoautotrophic Microorganisms from a Diffuse  
Flow Hydrothermal Vent at EPR 9° North using  $^{13}\text{C}$  DNA Stable  
Isotope Probing and Catalyzed Activated Reporter Deposition-  
Fluorescence *in situ* Hybridization**

By

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## ***Abstract:***

At deep-sea hydrothermal vents chemolithoautotrophic microbes mediate the transfer of geothermal chemical energy to higher trophic levels. To better understand these underlying processes and the organisms catalyzing them, this research used DNA Stable Isotope Probing (SIP) combined with Catalyzed Activated Reporter Deposition-Fluorescence in situ Hybridization (CARD-FISH) to identify the microorganisms chemoautotrophically supporting the food web at a diffuse flow hydrothermal vent. Both anaerobic and aerobic shipboard incubations containing various augmented electron donor and acceptor species showed that Epsilonproteobacteria were the dominant chemoautotrophs with greater than 70% of the cells counted within the first 24 hours.  $^{13}\text{C}$  DNA SIP identified unique organisms not previously characterized from low temperature diffuse flow venting: green sulfur bacteria (Chlorobi-like organisms) possibly utilizing photoautotrophy, aerobic *Lutibacter litoralis*-like organisms growing under anaerobic conditions, and Epsilonproteobacterial *Thioreductor* sp. at temperatures above maximum known tolerances. This research illustrates both the promise and pitfalls of the SIP technique applied to hydrothermal systems, concluding that timing of the incubation experiments is the critical step in eliminating undesired  $^{13}\text{C}$  labeling. These results set the stage for a more thorough future examination of diffuse flow microorganisms by presenting interesting questions that second generation experiments could be designed to answer.

## ***History and Background:***

### ***Hydrothermal Vent Systems***

Deep-sea hydrothermal vents were first discovered 32 years ago on the spreading center of the Galapagos ridge (Lonsdale, 1977, Corliss et al., 1979). They are commonly located along mid-ocean ridges where oceanic plates are spreading apart due to an underlying magma chamber in the Earth's mantle. At these locations, cold, deep-sea water is penetrating the highly porous, newly formed oceanic crust, and through seawater-rock interactions at depth and at high temperature and pressure it is being transformed into a highly reduced, superheated, and acidic fluid, also referred to as hydrothermal fluid. The hydrothermal fluid that is highly enriched in reduced chemical species, such as  $H_2$ ,  $H_2S$ ,  $Fe^{2+}$ , travels back to the seafloor where it is emitted into the deep ocean in basically two modes: as focused flow or diffuse flow.

At focused flow, fluids are emitted undiluted at high temperatures (to 400°C) through so-called black smoker chimneys. At diffuse-flow vents, hydrothermal fluid mixes in the subseafloor with various amounts of oxygenated seawater and exits the seafloor at lower temperatures, often between 10°C to 60°C. Over the years, hydrothermal vent science has focused mainly on studying the hot focused black smoker venting, and less attention has been paid to diffuse-flow vents. However, it is the mixing zones in the upper crust that have the potential to harbor abundant microbial life due to the simultaneous presence of oxidized and reduced chemical species creating chemical disequilibria that can be harnessed by a diverse array of metabolically versatile microorganisms. At present information on the identity of the microbes living in this vast system and their activities and contribution to overall biomass production at vents is limited. This study addresses the diversity and identity of chemoautotrophic microbial communities associated with diffuse flow vents.

## ***Diffuse Flow Venting***

Diffuse flow chemistry depends initially on the mixing events between hydrothermal fluid and entrained seawater. It is later affected by conductive cooling of the fluid as it travels through the subsurface (Cooper et al., 2000), the precipitation and/or dissolution of mineral species (sulfides, silica, anhydrites) (James and Elderfield, 1996), and redox transformation by microorganisms (Nunoura and Takai, 2009). The fluid contains both the reduced species contributed by high temperature vent fluids ( $H_2$ ,  $H_2S$ ,  $CO_2$ ) and the oxidized species ( $NO_3$ ,  $O_2$ ) from ocean bottom water. These species exist in a chemical disequilibrium that is harnessed by microorganisms, which catalyze energy-producing reactions in the direction of chemical equilibrium.

Because of its origins in the subsurface the diffuse fluid contains microbes representative of the various microenvironments within which the flow has traveled (Holden et al., 1998). That is why several studies have suggested diffuse fluid to be an excellent proxy for direct measurement of subsurface microorganisms. Summit and Baross (2000) found hyperthermophiles of the genus *Thermococcus* in the low temperature diffuse flow at Juan De Fuca Ridge and classified them as “microbial tracers,” meaning they are indicators of a hot subsurface biosphere. Their research further showed that populations of *Thermococcus* from the diffuse flow, representing the subsurface, were phylogenetically distinct from related genera found in black smoker sulfide chimneys, implying that speciation had occurred between the organisms inhabiting these two environments, and that the subsurface probably constitutes a separate habitat with its own distinct biota.

Huber et al. (2002, 2003) characterized bacterial and archaeal populations originating from the subsurface using diffuse flow fluids as the sampling source. They were successful in identifying unique subseafloor phylotypes from Epsilonproteobacteria, Methanococcales, and Thermoplasmata classes. They also showed that as the chemistry and temperature of the diffuse flow hydrothermal fluids changed over time so did the composition of the associated microbial communities. The classes of microorganisms found in the Huber et al. (2002, 2003)

study were later confirmed by a tag sequencing analysis of two diffuse flow sites (Huber et al. 2007). The tag sequencing results also listed several novel divisions (Chlorobi and the filamentous anaerobic phototroph Chloroflexi for example), presenting further questions about the link between the subsurface and diffuse flow venting. Higashi et al. (2004) drilled a bore-hole near the Suiyo Seamount hydrothermal vent field creating an artificial orifice which vented fluids that were subsequently sampled using microcolonizers (artificially assembled pieces of surface designed to encourage the growth of bacterial communities). These microcolonizers revealed novel phylotypes within the Epsilonproteobacteria bacterial subdivision as well as possible photosynthetic Alphaproteobacteria.

It is suggested that the zone of habitability within the ocean subsurface can be as deep as a few kilometers, but the current extent of life below hydrothermal systems is unknown (Colwell, 2001). These zones are almost certainly dominated by hyper/thermophilic anaerobic bacteria due to the high temperatures and anoxic reduced conditions of the hydrothermal fluid (Takai and Horikoshi, 1999). But because subsurface mixing creates conditions for many different microbes with varied metabolisms, it is also possible that the organisms found to colonize surfaces of basalt, the symbionts of metazoans, or free-living hydrothermal bacteria may have their source populations in the subsurface. To further study subsurface microorganisms this research chemically and thermally simulated subsurface microenvironments using diffuse flow fluid in order to identify important microbial communities.

### ***Microbiology at Vents***

Microorganisms are critical to almost all of the Earth's living processes, and have been discovered in extraordinary places having adapted to the planet's most extreme environments. Deep-sea hydrothermal vents are one of the most extreme environments known, and the communities of microorganisms that thrive there have evolved to harness the inhospitable conditions of reduced sulfur species, methane, hydrogen, and highly variable temperatures to their biological advantage. To study these organisms scientist have traveled to the depths of the world's oceans

with experimental techniques developed specifically for hydrothermal systems. However a recent analysis by Pettit (2010) pointed out that due to the versatility of hydrothermal microorganisms it is not always necessary to recreate the extreme environments from which they originate in order to study them.

It is known that at hydrothermal vents microorganisms are the primary producers converting inorganic carbon into cellular biomass that almost exclusively fuels higher trophic levels. These communities vary spatially within the vent environments (temperature and chemical gradients) and temporally with respect to colonization (Page et al., 2008). Communities of microorganisms have been found and studied on solid surfaces adjacent to vent sites (Longnecker and Reysenbach, 2001), in the walls of black smoker chimneys (Zhou et al., 2009), in low temperature venting not associated with black smokers (Perner et al., 2007), and coexisting within vent metazoans as symbiotic partners (Arndt et al., 2001).

Despite the evolution of communities in separate microenvironments patterns have emerged that indicate important overall trends. Chemoautotrophy based on sulfur oxidation has previously been identified as a major energy generation process for vent microorganisms (Emerson et al., 2007), but recent studies are discovering a larger variety of redox couples (hydrogen, iron, manganese, methane) utilized as energy sources by bacteria and archaea (Hirayama, et al., 2007). A specific subdivision of bacteria (Epsilonproteobacteria) has emerged several times in various studies as the dominant class of microorganisms in vent communities (Longnecker and Reysenbach, 2001, Lopez Garcia et al., 2003, Konstantinos et al., 2006).

### ***Chemoautotrophy at Vents***

In contrast to the important role of photoautotrophy in the terrestrial biosphere, a unique feature of hydrothermal vent microbial communities is their reliance on chemoautotrophy for energy production, carbon fixation, and the transfer of carbon through food webs. Chemoautotrophy is the derivation of energy from the oxidation of reduced inorganic chemicals. This energy is then used to fix inorganic carbon.

Microorganisms harness chemical energy by catalyzing redox reactions that combine available electron donating species with electron accepting species. Aerobic chemoautotrophy takes place in the presence of O<sub>2</sub>, which acts as the terminal electron acceptor. Anaerobic chemoautotrophy takes place in the absence of oxygen and can use multiple electron acceptors (e.g. NO<sub>3</sub>, SO<sub>4</sub>, Fe<sup>3+</sup>).

Several forms of vent chemoautotrophy are known, and they generally group with the types of reactions carried out by populations of microorganisms. Sulfur-oxidizing organisms (such as *Thiomicrospira*, *Thiobacillus*) acquire energy by coupling the redox potential of reduced sulfur compounds (thiosulfate, H<sub>2</sub>S) as electron donors with a variety of electron acceptors (O<sub>2</sub> in an oxic environment, NO<sub>3</sub><sup>-</sup>, SO<sub>4</sub><sup>2-</sup> in anoxic environments). Hydrogen oxidation is a second important mechanism for energy generation in a number of hydrothermal vent microorganisms such as *Sulfurovum* sp. and *Sulfurimonas paralvinellae* (Yamamoto et al., 2010, Takai et al., 2006). Other chemoautotrophs use iron chemistry in their redox couples oxidizing elemental sulfur with Fe<sup>3+</sup> or oxidizing Fe<sup>2+</sup> with nitrate for example (Emerson et al., 2007, Hodges and Olson, 2009). There are several other mechanisms for energy generation that could have substantial roles in the subsurface biosphere: carboxytrrophy, manganese oxidation, methane oxidation, ammonia oxidation (Nakagawa and Takai, 2008). Part of gathering more information on the functioning of chemoautotrophy in the subsurface is not simply identifying which organisms and metabolic mechanisms are present, but identifying which are predominantly active in specific habitats and niches.

Certain bacterial classes utilizing chemoautotrophy have emerged in recent research as being predominant in vent environments. Gammaproteobacteria are commonly found as chemoautotrophic endosymbionts within vent fauna (Urakawa et al., 2005, Nakagawa and Takai, 2008) and also occur as free-living chemoautotrophs such as *Thiomicrospira* and *Beggiatoa* (Loesekann et al., 2007). Their importance in sulfidic environments was recognized before their discovery at hydrothermal vents, and it was hypothesized that they would also be the predominant microorganisms on vent surface structures (Polz and Cavanaugh, 1995). Gammaproteobacteria genes have been discovered in the tag sequencing



and 16S surveys of various vent habitats, including snapshots of subsurface populations (Huber et al., 2007, Perner et al., 2007), but more evidence is mounting that a separate class of proteobacteria from the subdivision Epsilonproteobacteria could predominate other deep sea vent habitats.

### ***Epsilonproteobacteria***

A recent review on Epsilonproteobacteria characterized them as “globally ubiquitous in modern marine and terrestrial ecosystems... [which] have had a significant role in the biogeochemical and geological processes throughout Earth’s history” (Campbell et al., 2006). Despite their potential biogeochemical relevance they are arguably the most poorly characterized subdivision of Proteobacteria with most reports suggesting their global importance published only in the last decade (Collado et al., 2009, Engel et al., 2003, Grote et al., 2008). Hydrothermal vent research has helped to expand the breadth of information about Epsilonproteobacteria because of their prevalence in vent taxonomic surveys. For example, they have been found in mats on basalt and chimneys (Lopez-Garcia et al., 2003), in diffuse vent fluids (Huber et al., 2003), in association with vent metazoans (Urakawa et al. 2005), and within the vent subsurface (Kimura et al., 2003). Their predominance in vent samples has led to the suggestion that deep-sea hydrothermal systems harbor the most diverse communities of Epsilonproteobacteria on Earth (Campbell et al., 2006).

The presence of Epsilonproteobacteria in vent environments is likely a result of their metabolic versatility in being able to access electrons from a variety of energy sources coupled with diverse electron acceptors. Vent-associated Epsilonproteobacteria have been associated with the oxidation of reduced sulfur compounds, hydrogen, organics, formate, and fumarate for energy derivation when combined with multiple electron acceptors such as nitrate, oxygen, and oxidized sulfur compounds. It is postulated that they may also oxidize carbon monoxide, iron, manganese, arsenic or selenium (Campbell et al., 2006). Representatives of the subdivision have been associated with autotrophic, mixotrophic, or heterotrophic growth. Because of this metabolic flexibility Epsilonproteobacteria can be the primary colonizers in diffuse flow environments by quickly growing at the interface

between new sulfidic and oxygenated zones utilizing this couple to their energetic advantage (Taylor et al., 1999).

Several vent research studies have found Epsilonproteobacteria to be the dominant microbial group in hydrothermal vent samples. Fluorescence *in situ* Hybridization (FISH) studies of the Lilliput low temperature venting area found a majority of hybridized cells were Epsilonproteobacteria (Perner et al., 2007). Microcolonizers in the area next to the ‘Tour Eiffel’ vent were almost entirely dominated by Epsilonproteobacteria (Lopez-Garcia et al., 2003). A five-day incubation with an *in situ* growth chamber deployed at Snake Pit in the Mid Atlantic (70°C) suggested that 40% of the sequences of a 16S rRNA clone library were Epsilonproteobacteria (Reysenbach, 2000). Vent-associated Epsilonproteobacteria have now been cultivated, yielding a wealth of new information about their metabolism and growth requirements (Takai et al., 2003). These data suggest that these bacteria are central to primary production at hydrothermal vents and generate biomass that is an important foundational component of the trophic web at these sites.

### ***Study Site: Crab Spa diffuse flow vent at East Pacific Rise (EPR) 9°N***

Since the early 90’s the hydrothermal vent field at EPR 9° North has been the subject of many investigations (Von Damm, 2000, Campbell and Cary, 2004, Santelli et al., 2008, Mullineaux, et al., 2010). The Crab Spa vent, located in the northern area (Tika Region) of the 9° North vent field, is a thermally stable (~30°C relatively constant outflow temperature over two years of sampling) diffuse flow vent with high concentrations of free sulfide (~1mM) associated with a high temperature venting system nearby (Tika). Crab Spa contains a well-defined orifice that has remained intact over two years making sampling at the site straightforward and easily reproducible. These factors combine with the fact that the area is regularly visited by scientific vessels to make Crab Spa an ideal choice for conducting research on diffuse flow vent microorganisms.

## **Materials and Methods:**

**Cruise Sampling:** Samples used in this research were obtained aboard the *R/V Atlantis* using the Alvin Submarine between October 28<sup>th</sup> and November 5<sup>th</sup>, 2008 on the AT15-38 cruise. Hydrothermal fluid from the Crab Spa site was collected for all experiments using 6 Titanium Major Pairs stored aboard the *Atlantis*. Fluid was collected in series with the collection nozzle of each Major Pair being placed in the same area of the Crab Spa orifice each time.

**Fluid Incubations:** For these experiments fluid collected sequentially from 6 individual Titanium Major Pairs was pooled to create the starting fluid used to set up each incubation. Incubations were performed under 3 chemical conditions hereafter referred to as (1), (3), and (5):

- |   |  |
|---|--|
| 1) No additions to the fluid  | (Headspace N <sub>2</sub> /CO <sub>2</sub> /O <sub>2</sub> ) |
| 3) + 1mM Nitrate (KNO <sub>3</sub> ) + 1mM Thiosulfate (Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> ) | (Headspace N <sub>2</sub> /CO <sub>2</sub> )                 |
| 5) + 1mM Nitrate (KNO <sub>3</sub> )  | (Headspace H <sub>2</sub> /CO <sub>2</sub> )                 |

Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> and KNO<sub>3</sub> were added to sterile incubation bottles from 1M sterile stock solutions prior to the addition of vent fluid. Headspaces were flushed after fluid addition and stoppers applied with either N<sub>2</sub>/CO<sub>2</sub> (80%/20%) or H<sub>2</sub>/CO<sub>2</sub> (80%/20%) for a minimum of 5 minutes. In addition to the flushing, condition (1) had 12ml of air added to its headspace by injection using a 0.2 micron filter for a final O<sub>2</sub> concentration of 2%. The final addition to all conditions before placing them at the appropriate temperatures was NaH<sup>13</sup>CO<sub>3</sub> label to an amount of 30% total bicarbonate concentration from a 0.2M stock solution. 30% of total bicarbonate was calculated based on a previous experiment on the fluid (experiment not shown) to be 580μl of 0.2M NaH<sup>13</sup>CO<sub>3</sub> per 50ml of fluid. Condition (1) bottles (1L Pyrex) were incubated with 950ml of fluid and 112ml of headspace for 72 hours and 144 hours in 30°C water baths. Condition (3) bottles (0.5L Wheaton) were incubated with 500ml of fluid and 87ml of headspace for 48 and 96 hours at two temperatures (one set of bottles in a 30°C water bath and a second set in a 50°C water bath). Condition (5) bottles (1L Pyrex) were incubated with 500ml of fluid and 562ml of headspace for 48 and 96 hours at two temperatures (30°C and

50°C). Individual incubation bottles were harvested at their appropriate time points by releasing the seal on the bottle and syringing the contents through a 0.2 micron Sterivex filter which was stored at -80°C until DNA extraction could be performed.

**Cell Fixation and Filtration:** Incubation fluid was taken from the 96 hour sealed incubation bottles by syringe at intervals of 24, 48 and 96 hours for fixation with formaldehyde. Fixation was performed by adding 5ml of fluid with 600µl of 37% formaldehyde and allowing the tubes to remain at 4°C for a minimum of 12 hours but no more than 24 hours. The fixed cells were then diluted 1:5 with 0.2 micron filtered seawater. 25mm 0.2 micron GTTP Millipore filters were used in conjunction with a 12x25mm filtration manifold (Millipore) to produce 4 filters per sample (1ml filtered, 2ml filtered, 5ml filtered, 10ml filtered). Filters were washed with 5ml of sterile water, air dried for 15 minutes minimum, and placed at -20°C for storage.

**DNA Extraction:** DNA was extracted from each Sterivex filter by dissecting each filter unit and placing one half of the dissected filter into each of two 2ml cryotubes. 1ml of DNA extraction buffer (100mM Tris-HCL pH 8.0, 100mM NaEDTA pH 8.0, 100mM phosphate buffer pH 8.0, 1.5M NaCl, 1% CTAB) was added to each tube followed by the addition of 20µl of fresh proteinase-K solution (10mg/ml in 0.1M Tris-HCl pH 8.0, 0.05M NaEDTA pH 8.0) and 20µl of fresh lysozyme solution (100mg/ml in 0.1M Tris-HCl pH 8.0, 0.05M NaEDTA pH 8.0). Tubes were incubated at 37°C for 30 minutes. 100µl of filter sterilized SDS (10% solution in water) was added and incubation of the tubes continued for 4 more hours at 65°C. The tubes were then filled to the top with phenol:chloroform:isoamyl alcohol (25:24:1, pH 8.0), vortexed, and centrifuged at 3000rpm for 10 min. The top layer (aqueous layer) was then transferred to a clean 2.0ml tube and filled again with phenol:chloroform:isoamyl alcohol. The new tube was vortexed, centrifuged at 3000rpm for 10 min, and the top layer transferred to a third clean 2.0ml tube. The third wash was performed with 100% chloroform, vortexed, spun down at 3000rpm for 10 min and the top layer transferred to a 50,000MW rose-colored Amicon Filter unit (Millipore). The filter units were centrifuged at maximum speed to concentrate

the DNA on the filter and in the 15µl reservoir at the base of the Amicon. The filter was washed with sterile DNA grade water twice, and centrifuged at maximum speed to remove wash water. 250µl of sterile DNA grade water was pipetted down the sides of the filter to resuspend the DNA, the filter was inverted and placed over a clean microfuge tube, and centrifuged at low speed to deposit the DNA/water solution in the microfuge tube.

***DNA Quantification (PicoGreen Assay):*** A set of standardized DNA solutions were made using 50, 20, 10, 2, and 1 ng/µl DNA stock solutions (1µl DNA solution, .5µl PicoGreen (Invitrogen), and 198.5µl of DNA/RNA free TE). Sample DNA solutions were made using the same formula. Standards and samples were analyzed in 200µl cuvettes using a Biorad Versa Fluor Fluorometer on the HI GAIN setting. A standard curve was produced on an Excel spreadsheet from the 5 standards and the linear equation used as the basis for calculating the sample concentrations from the fluorometer reading. The protocol was based on the publication by Ahn et al. (1996).

***Moving-Wire DNA Mass Spectrometry:*** Samples of total extract DNA (~20ng/µl) were loaded in triplicate (0.5µl per sample) onto a Moving-Wire Analyzer courtesy of Ann Pearson at Harvard University (Analyzer described in Sessions et al. 2005). The three runs were averaged for each sample, and using a data analysis package developed by Ann Pearson computer software determined the  $\delta^{13}\text{C}$  value for each extract.

***Stable Isotope Probing (SIP) Centrifugation:*** For SIP centrifugation the 2007 Neufeld et al. protocol was used as a reference. 500ng-1µg of sample DNA was combined with 1.80g/ml CsCl and gradient buffer (0.1M Tris-HCL pH 8.0, 0.1M KCl, 1mM EDTA pH 8.0) to form a final solution with average density of 1.725g/ml. The solution was applied to a 5.1ml Opti-Seal polyallomer Beckman centrifugation tube filling it to the neck of the tube. Tubes were massed to within 10mg of each other to balance out the centrifuge, sealed with Opti-Seal stoppers, and placed in a Beckman VTi62.5 rotor. The rotor was spun in a L-8 series ultracentrifuge under vacuum for

48 hours, at 20°C, 44,100rpms, and no brake. A control tube containing 1.5µg of <sup>12</sup>C *Sulfurimonas d.* DNA and 1.5µg of <sup>13</sup>C *Sinorhizobium meliloti* DNA (courtesy of Josh Neufeld) was always run alongside all sample tubes.

***SIP Fractionation and Precipitation:*** Once removed from the centrifuge the tubes were quickly placed in a clamp and the surface of the tube cleaned with isopropanol pads. A hole was carefully poked in the base of the tube using a 23 gauge needle under which sat 12 collection microfuge tubes. Into the top of the tube a needle was placed connected to a syringe pump with sterile milli-Q water in the syringe. The water was pumped into the top of the tube at a rate of .41ml/min and drops were collected into the 12 tubes at one fraction/min. An AR200 digital refractometer was used to collect data on the specific density of each individual fraction. 3µl of 20mg/ml glycogen in DNA grade water was then added to each tube along with 800µl of PEG-6000 (30% PEG-6000, 1.6M NaCl). The tubes sat in the dark overnight and were then placed in the microcentrifuge at maximum speed for 30 min. The liquid was aspirated from the visible white pellet of glycogen/DNA and washed with 80% Ethanol. The tubes were spun at maximum speed for 10 min and the wash liquid aspirated away. Pellets were allowed to air dry for no more than 15 min and resuspended in 30µl of PCR grade water. Concentration of each fraction was determined by the PicoGreen assay described above.

***Oligonucleotide Primers and Primer Design:*** Two sets of primers were used in these experiments. One previously published set of forward and reverse primers for capturing bacterial 16S rRNA sequences: 341f (5'-CCTACGGGAGGCAGCAG-3') forward primer and 907r (5'-CCGTCAATTCMTTGTGAGTTT-3') reverse primer (Muehling et al., 2008). A second primer pair was designed for this study to capture Epsilonproteobacteria sequences for sequencing and denaturing gradient gel electrophoresis (DGGE): E1f (5'-AGCGTTAYTCGGAATCACTGG-3') forward primer and E2r (5'-CCCCGTCTATTCTTTGAGTTTT-3') reverse primer. The primers were designed with Sequencer using 10 sequences from vent Epsilonproteobacteria in this study in addition to *Helicobacter* and *Sulfurospirillum*. The primers were checked to total available Epsilonproteobacterial sequence space using probeMATCH with E1f and E2r having a 62% and 55% Epsilonproteobacteria

match, respectively, and less than 1% match to any sequence outside of Epsilonproteobacteria. E1f and E2r have T<sub>m</sub>'s of 55°C and 53°C respectively and an annealing temperature as a pair in PCR reactions of 62.5°C for maximum specificity. When a GC clamp was required for DGGE PCR the clamp used (5'-CGCCGCGCGCGCGGGCGGGGCGGGGGCACGGGGGC-3') was attached to the forward primer (Ferris et al., 1996).

***Polymerase Chain Reaction (PCR):*** Initial DGGE PCR was performed in a 50µl volume containing 25µl of GoTaq Hotstart Master Mix Green (Promega), 1µl of 25µM forward primer, 1µl of 25µM reverse primer, 1µl of template (1-20ng of DNA), and 22µl of PCR water (Teknova). The PCR was performed on a Bio-Rad thermal cycler using a touchdown thermal cycling program with the following steps: denaturation at 94°C for 2 min; 20 touchdown cycles of denaturation (94°C for 30 sec), annealing (65°C-45°C for 30 sec decreasing 1°C every cycle), and extension (72°C for 1 min); 20 standard cycles of denaturation (94°C for 30 sec), annealing (51°C for 30 sec), and extension (72°C for 1 min); final extension (72°C for 5 min). PCR reactions were run on a 1% agarose gel to confirm existence of product. Sequencing PCR was performed in a 100µl volume containing 50µl of GoTaq Hotstart Master Mix Clear (Promega), 2µl of 25µM forward primer, 2µl of 25µM reverse primer, 1µl of template (eluted gel fragments in TE pH 8.0, varying concentrations of DNA), and 45µl of PCR water (Teknova). The PCR program was a standard 15 cycle amplification: denaturation at 94°C for 2 min, 15 cycles of denaturation (94°C for 30 sec), annealing (51°C (341f/907r) or 62.5°C (E1f/E2r) for 30 sec), and extension (72°C for 1 min); final extension (72°C for 5 min).

***Denaturing Gradient Gel Electrophoresis (DGGE):*** PCR products were loaded (volume depended on quantity of product for each set of reactions but SIP fraction sets were always loaded with the same volume for every fraction in the set) onto a 6% polyacrylamide gel with a linear gradient of denaturants (urea and formamide) increasing from 20% at the top to 70% at the bottom. A Bio-Rad DGGE system was used to run the gels for 14hours at 100 V at 60°C in 1x TAE buffer. The gels were removed from the system and placed on an Invitrogen Safe Imager. SYBRGold

(Invitrogen) at 1x concentration in milli-Q water was poured over the gels and allowed to bind DNA in the dark for 30 min. An image of each exposed gel was taken with a Canon 50D SLR camera and gel bands excised.

***Gel Band Reamplification:*** Excised gel bands were eluted in 50µl TE pH 8.0 overnight. PCR was performed in a standard 100µl reaction with a 15 cycle program as described previously but using a forward GC-Clamp primer. 1% agarose gel electrophoresis was used to quantify reamplification products. A second DGGE was run using a 20%/70% gel as described above. A total DNA extract GC clamp PCR for each SIP condition was used as a control lane to identify the reamplified bands. Bands were excised and eluted as described then used as template for sequencing PCR. The second round of DGGE was to ensure a pure band containing no contaminating neighbor bands.

***DNA Sequencing:*** Sequencing was performed by Beckman Coulter Sequencing (Danvers, MA) in 96-well plates with both a forward primer reaction and a reverse primer reaction for each DGGE gel band (341f/907r or E1f/E2r). Sequences from each primer direction were combined and edited using Sequencer to form the consensus sequence for each gel band.

***HRP-Probes:*** Probes were ordered from Thermo Fisher Scientific (Ulm, Germany) and all conjugated on the 5' end with Horseradish Peroxidase (HRP). The final probes for general Bacteria consisted of a 1:1:1 mixture of EUB338I (5'-HRP-GCTGCCTCCCGTAGGAGT-3')(Amann et al., 1990), EUB338II (5'-HRP-GCAGCCACCCGTAGGTGT-3')(Daims et al., 1999), and EUB338III (5'-HRP-GCTGCCACCCGTAGGTGT-3')(Daims et al., 1999). The final probes for Epsilonproteobacteria consisted of a 1:1 mixture of EPS914 (5'-HRP-GGTCCCCGTCTATTCCTT-3')(Loy et al., 2007) and EPSY549 (5'-HRP-CAGTGATTCCGAGTAACG-3')(Lin et al., 2006). The probe for Gammaproteobacteria was GAM42a (5'-HRP-GCCTTCCCACATCGTTT-3') with competitor cGAM42a (5'-GCCTTCCCACCTTCGTTT-3')(Manz et al., 1992).



### ***Catalyzed Activated Reporter Deposition-Fluorescent in situ Hybridization***

**(CARD-FISH):** Filter pieces were embedded using ~30µl 0.1% low melting point agarose and allowed to dry face up at 37°C in an open petri dish. Filters were dehydrated in 96% ethanol causing them to detach from the surface of the petri dish, and then moved to a piece of blotting paper to dry. Whole filters were permeabilized in petri dishes containing 10ml of freshly made lysozyme solution (10mg/ml lysozyme, 0.1M Tris-HCl pH 7.5, 0.05M EDTA) for 1 hour at 37°C. Each filter piece was washed twice in 50ml of milli-Q water and then transferred to a petri dish of 0.1M HCl for 1 minute. The filters were then transferred to a petri dish of 0.01M HCl for 10 min. Filter pieces were washed twice again with 50ml of milli-Q and dehydrated by 30 sec washes in successive dishes of 50% ethanol, 80% ethanol, and finally 96% ethanol. Filter pieces were dried on blotting paper and stored at -20°C until hybridization. Probes for Bacteria were hybridized in a buffer of 35% formamide and probes for Epsilonproteobacteria and Gammaproteobacteria in a buffer of 55% formamide (hybridization buffer: 10% Dextran Sulfate, 900mM NaCl, 20mM Tris-HCl pH 8.0, 0.05% Triton-X, 1% Blocking Reagent, and either 55% or 35% formamide). 6µl of HRP probe working solution (50ng/µl) were added to 600µl of hybridization buffer in amber colored microfuge tubes containing at maximum 8 filter pieces. The tubes were incubated on a rotator in a 35°C hybridization oven for 3 hours. Filter pieces were then washed in pre-warmed (37°C) washing buffer (20mM Tris-HCl pH 7.5, 5mM EDTA, 0.01% SDS, and 13mM NaCl for Epsilon and Gamma probes; 145mM NaCl for Bacterial probe) at 37°C for 20 minutes (mildly shaking the wash tube every 5 minutes). Filter pieces were transferred from the washing buffer to a 50ml tube of 1x PBS containing 0.05% Triton-X (PBS-T) and let stand at room temperature for 20 minutes. They were further washed with 50ml of 1x PBS before tyramide amplification. A 100x peroxide mixture was made by adding 5µl of fresh 30% H<sub>2</sub>O<sub>2</sub> to 1000µl of 1x PBS (the same PBS used for washings). 10µl of peroxide mix was placed in 1ml of Amplification Buffer (10% Dextran Sulfate, 2M NaCl, 0.1% Blocking Reagent in 1x PBS) along with 2µl of tyramide conjugated with Alexa dye 488 (A<sub>488</sub>). Filter pieces were dried on blotting paper and added to the amplification mixture in a 1.5ml microfuge tube and

placed at 37°C in the dark for 30 minutes. Filter pieces were then washed with 50ml PBS-T followed by 2 washes with 50ml of milli-Q water. The filters were dipped in 96% ethanol and allowed to dry on blotting paper. They were mounted onto glass slides using a DAPI mix (1µg/ml DAPI – total DNA stain, 140µl/ml Vectarshield, 770µl/ml Cititfluor, in 1x PBS). A Zeiss Axioplan 2 microscope was used for the CARD-FISH microscopy (cell counting) and images taken using a 40x objective lens.

## ***Results:***

### ***<sup>13</sup>C Incubations:***

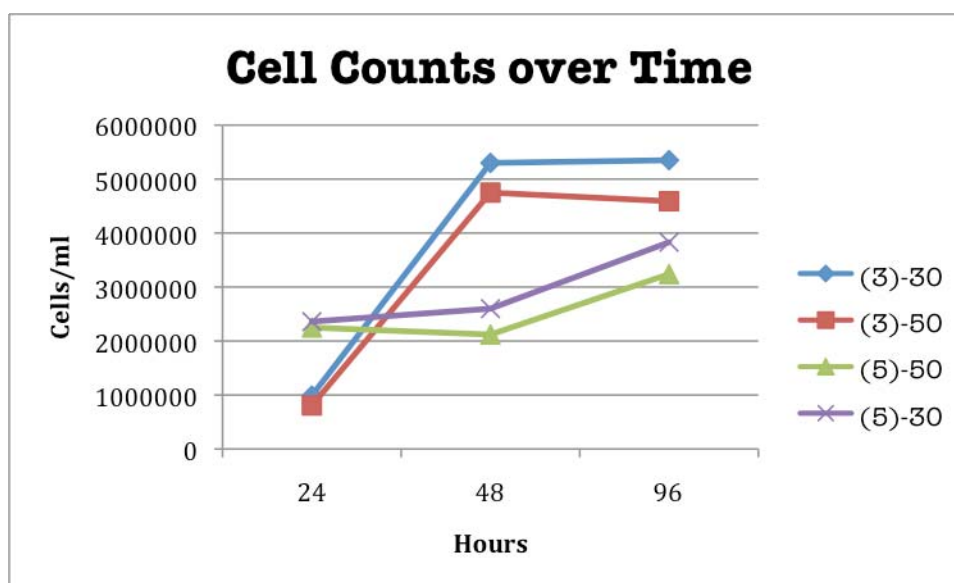
After the incubation bottles were setup, sealed, and gassed they were placed in water baths kept at either 30°C or 50°C depending on the experiment. The bottles were observed visually every 8-12 hours to monitor any changes. After 12 hours all incubations showed small signs of turbidity and the deposition of a film at the base of the bottle. After 24 hours incubations (3) and (5) at 30°C had formed a filamentous flocculent material that was sampled from the (5)-30-96hour bottle and observed under a field microscope to be “probable” elemental sulfur filaments (Wirsen et al., 2002). At 48 hours during the first harvesting of the (3) and (5) conditions the level of flocculent material had decreased slightly by approximately 30%, and by 72 hours it had disappeared entirely. At 144 hours the only experimental bottle left (condition 1) showed signs of a yellowish precipitate and a strong white biofilm that covered the bottom of the bottle.

### ***Cell Counts:***

DAPI cell counts were performed on the 1ml FISH filters for experiments at the 24 hour, 48 hour, and 96 hour stages (72 hours and 144 hours for condition 1). Pieces of each filter were mounted with DAPI stain directly onto glass slides without going through the CARD-FISH hybridization process for the purposes of total cell counting. Table 1 and Figure 2 show these counts (minimum of 1000 cell counted per filter).

	24 hours	48 hours	96 hours		72 hours	144 hours
(3)-30	$9.88 \times 10^5$	$5.30 \times 10^6$	$5.35 \times 10^6$	(1)-30	$1.85 \times 10^6$	$3.00 \times 10^6$
(3)-50	$8.06 \times 10^5$	$4.75 \times 10^6$	$4.59 \times 10^6$			
(5)-30	$2.25 \times 10^6$	$2.60 \times 10^6$	$3.83 \times 10^6$			
(5)-50	$2.36 \times 10^6$	$2.12 \times 10^6$	$3.24 \times 10^6$			CS= $6.15 \times 10^5$

**Table 1: DAPI cell counts.** Counts are in cells/ml of incubation fluid. The starting value is denoted by CS (Crab Spa) and stands for the cell count of the hydrothermal fluid coming straight out of the Titanium Major before incubation. 30 and 50 refer to 30°C incubation temperature and 50°C incubation temperature respectively and the nomenclature of (incubation)-temperature-time is used throughout the experiment {(1)-30-72 is abbreviated as (1)-72 and (1)-30-144 is abbreviated (1)-144}.

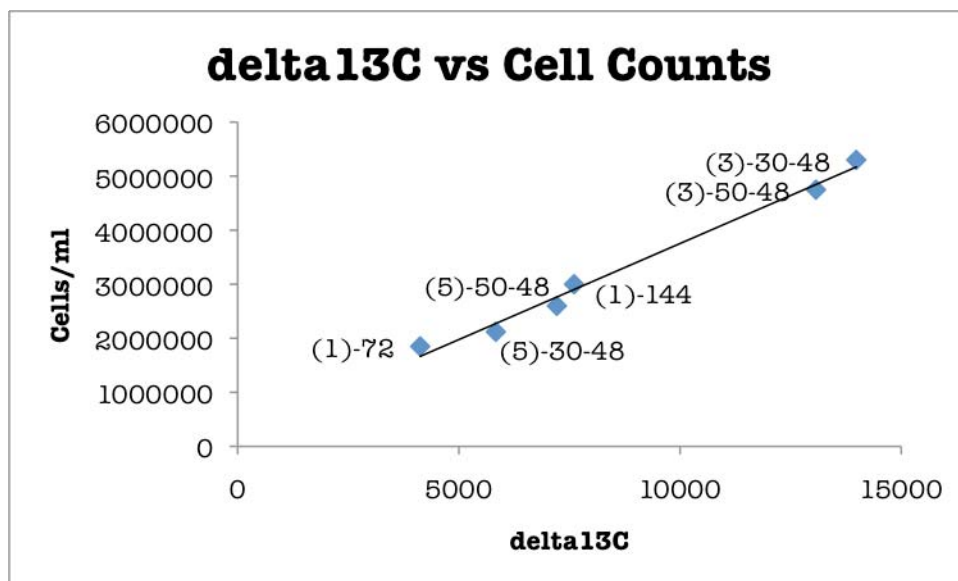


**Figure 2: Cell Count Graph.** A subset of the data in Table 1 graphed to show the difference between conditions (3) and (5) in approximate cell counts.

Growth and replication in condition (5) occurred initially at a faster rate than condition (3) for the first 24 hours independent of temperature. From 24 to 48 hours the replication rate of condition (3) increased as condition (5) hit a plateau. Temperature does not appear to be a major determinative factor in the number of cells produced although 30°C incubations did end the experiment with slightly more cells than their respective 50°C incubations.

### **$\delta^{13}\text{C}$ Data:**

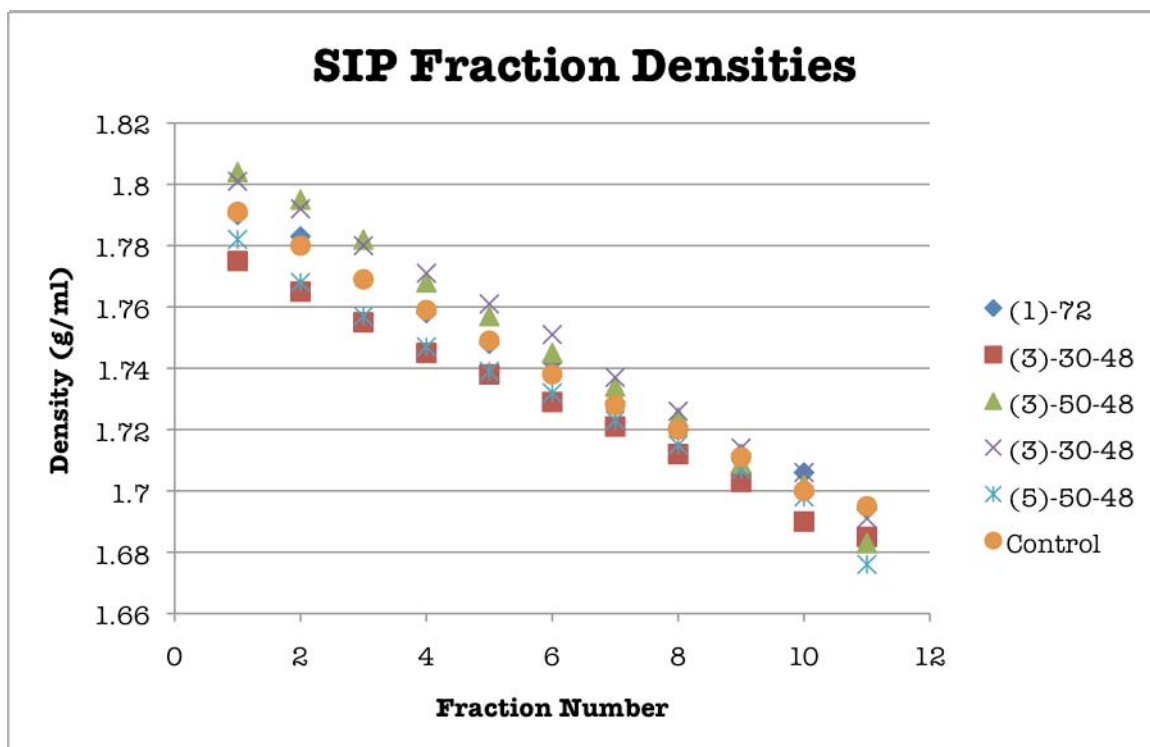
In order to assess how much  $^{13}\text{C}$  bicarbonate label had been taken up by chemoautotrophs and incorporated into DNA the following graph was made of  $\delta^{13}\text{C}$  values (moving-wire experiment) vs DAPI cell counts (Figure 3). The DNA extracted from each sample corresponds to the total DNA pool still present in the cells at the time of fixation.



**Figure 3:  $\delta^{13}\text{C}$  values plotted against DAPI cell counts.** The trend line indicates a linear relationship between  $^{13}\text{C}$  label incorporated into the DNA and the multiplication of cells over time.

### ***DNA Recovered from SIP fractionation:***

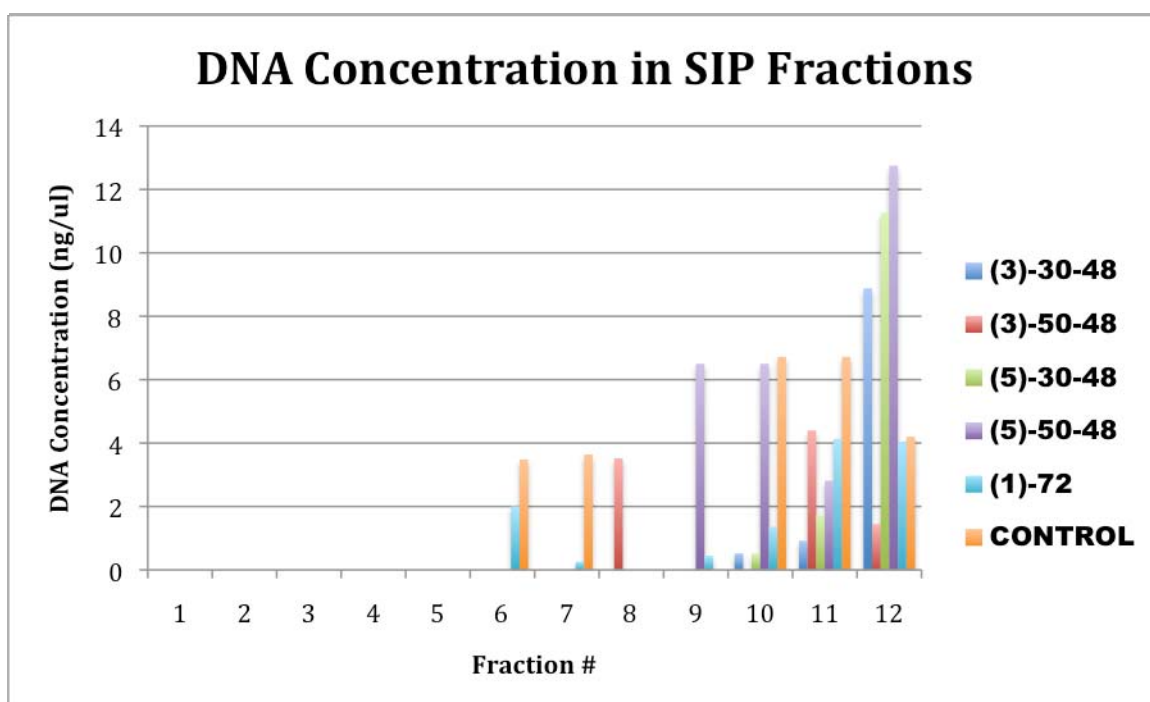
The density measurement of each individual fraction was taken as a control to ensure that separation had occurred in each centrifugation tube (Figure 4).



**Figure 4: Individual CsCl density measurements for SIP fractions.** The graph shows a predicted steady decline in the CsCl density of each fraction as fraction numbers increase. Fraction 1 represents the densest CsCl and therefore the heaviest DNA and fraction 11/12 represents the least dense fraction and therefore the lightest DNA. Fraction 12 is omitted from this plot because as the fractions are collected fraction 12 becomes partially mixed with water and therefore does not give an accurate reading of its true density within the centrifugation tube.

Once DNA from each fraction was precipitated with PEG-6000 the concentration of DNA was determined to further show a separation between light ( $^{12}\text{C}$ ) DNA and heavy ( $^{13}\text{C}$ ) DNA (Figure 5). The separation is not equally distinct in all incubations. This means that either the separation was not as successful in some tubes as in others or the nature of the  $^{13}\text{C}$  DNA labeling was not pronounced in incubations with less obvious separation.

Fraction #	(3)-30-48	(3)-50-48	(5)-30-48	(5)-50-48	(1)-72	Control
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	0
6	0	0	0	0	3.48	2.01
7	0	0	0	0	3.64	0.26
8	0	3.52	0	0	0	0
9	0	0	0.01	6.5	0	0.45
10	0.52	0	0.52	6.5	6.72	1.36
11	0.92	4.4	1.72	2.81	6.72	4.13
12	8.88	1.44	11.26	12.75	4.2	4.04



**Figure 5: DNA concentration of individual fractions.** A separation is clearly visible in the control fractions as well as (1)-72 and (3)-50-48. (3)-30-48, (5)-30-48, and (5)-50-48 show a smear of DNA from fraction 12 decreasing towards fraction 8. DNA is listed in a concentration of ng/ $\mu$ l. Fractions represent density changes from high to low going from fraction 1 to fraction 12.  $^{13}\text{C}$  DNA is found in the higher density fractions (smaller fraction numbers) while  $^{12}\text{C}$  DNA is found in lower density fractions (higher fraction numbers).

#### ***CARD-FISH cell percentages:***

CARD-FISH probes for Bacteria, Epsilonproteobacteria, and Gammaproteobacteria were used to determine the percentage of each microbial type in filters corresponding to incubation time points of 24, 48, and 96 hours (72

hours and 144 hours for condition 1). Positive and Negative filter controls for Epsilonproteobacteria (*Sulfurimonas d.*-positive, *E. coli*- negative), Gammaproteobacteria (*E. coli*- positive, *Sulfurimonas d.*-negative), and Bacteria (*E. coli*- positive, *Nitrosopulmilus m.*- negative) were used to test the efficacy of the probes used. Results for the cell counts of specific microbial classes are presented in a table in Table 6.

<b>EPSILON</b>			
CS	70.60%		
(1)-72h	85.10%		
(1)-144h	69.50%		
	<b>24hours</b>	<b>48hours</b>	<b>96hours</b>
(3)-30	99.90%	91.40%	90.00%
(3)-50	97.30%	95.40%	78.30%
(5)-30	74.90%	93.90%	91.00%
(5)-50	72.10%	50.40%	57.60%
<b>BACTERIA</b>			
CS	99.00%		
(1)-72h	98.00%		
(1)-144h	96.00%		
	<b>24hours</b>	<b>48hours</b>	<b>96hours</b>
(3)-30	93.00%	99.00%	98.80%
(3)-50	90.00%	99.00%	n/a
(5)-30	99.00%	97.00%	95.50%
(5)-50	100.00%	n/a	n/a
<b>GAMMA</b>			
CS	7.10%		
(1)-72h	2.00%		
(1)-144h	6.00%		
	<b>24hours</b>	<b>48hours</b>	<b>96hours</b>
(3)-30	0.00%	0.25%	2.00%
(3)-50	0.25%	1.00%	3.00%
(5)-30	0.00%	1.00%	3.00%
(5)-50	0.00%	0.00%	2.00%

**Table 6: Cell Count Percentages.** A minimum of 1000 DAPI cells (and 5 fields) were counted for each percentage. Percentages were calculated by dividing the number of positive  $A_{488}$  cells by the total number of DAPI cells in the same field. “n/a” stands for filters where the cells were too heavily degraded by the FISH process to count accurately.

Bacterial counts not being 100% in some cases suggests the presence of archaeal populations in the incubations. The Crab Spa vent fluid had an initial percentage of

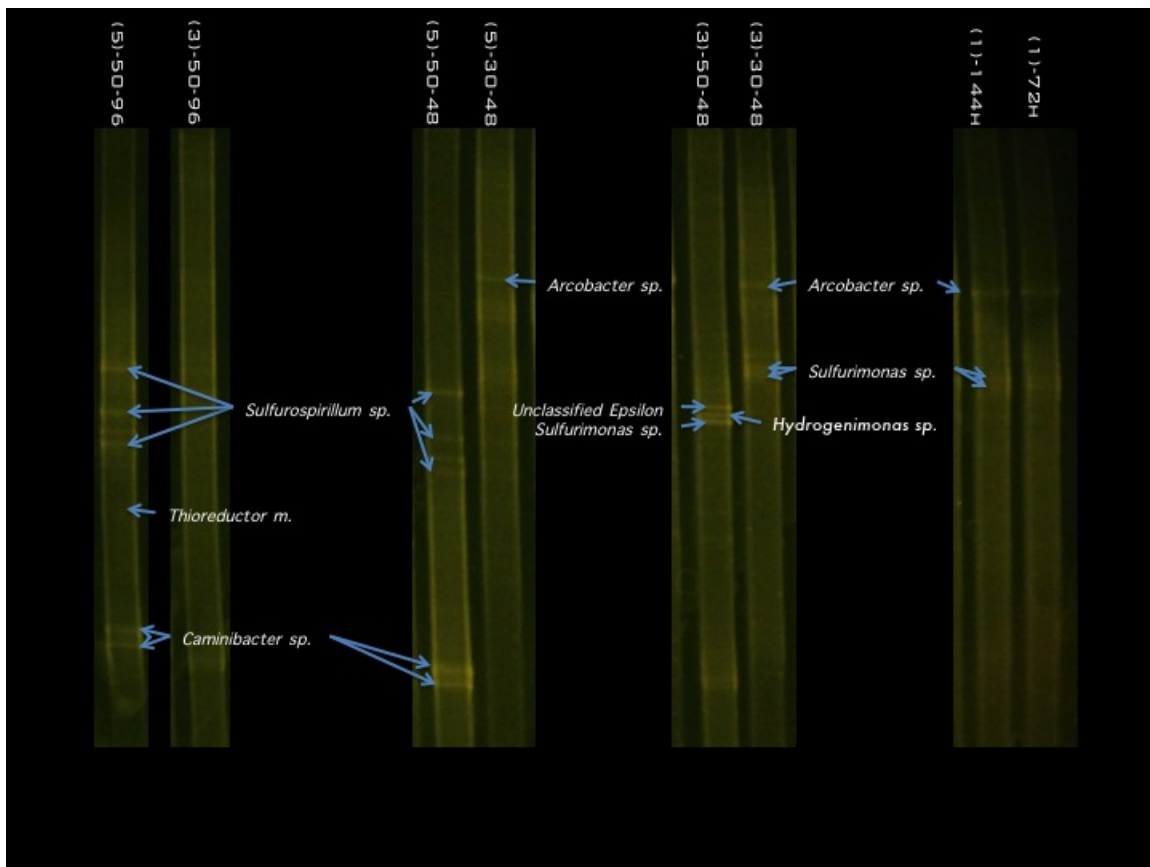
gammaproteobacteria at 7.1%, which dropped to uncountable levels after 24 hours. Later in every incubation Gammaproteobacterial cells began increasing in number.

***Epsilonproteobacteria identified in Total DNA Extracts:***

DGGE PCR was performed on each of the total extracts involved in this study using the E1f/E2r primer pair developed specifically to target Epsilonproteobacteria. The primer pair was tested with a temperature gradient PCR program against Gammaproteobacteria and Betaproteobacteria to determine an annealing temperature (62.5°C) that would specifically amplify Epsilonproteobacteria and not nonspecifically amplify other proteobacteria (data not shown). The Epsilonproteobacterial sequences were amplified from total extracts and run on DGGE gels in order to identify the major 16S rRNA sequences of Epsilonproteobacterial from individual incubation conditions (Figure 7).

*Arcobacter* sp. sequences were found in all 30°C incubations. *Sulfurimonas*, *Sufurospirillum*, and *Caminibacter* sequences appeared in multiple bands within the same total extract indicating that more than one representative of each genus could be present in these incubations. Both *Sufurospirillum*, and *Caminibacter* were the major bands seen in condition (5) at 50°C after 48 and after 96 hours of incubation. No dominant epsilonproteobacterial band was sequenced from the (3)-50-96 incubation.

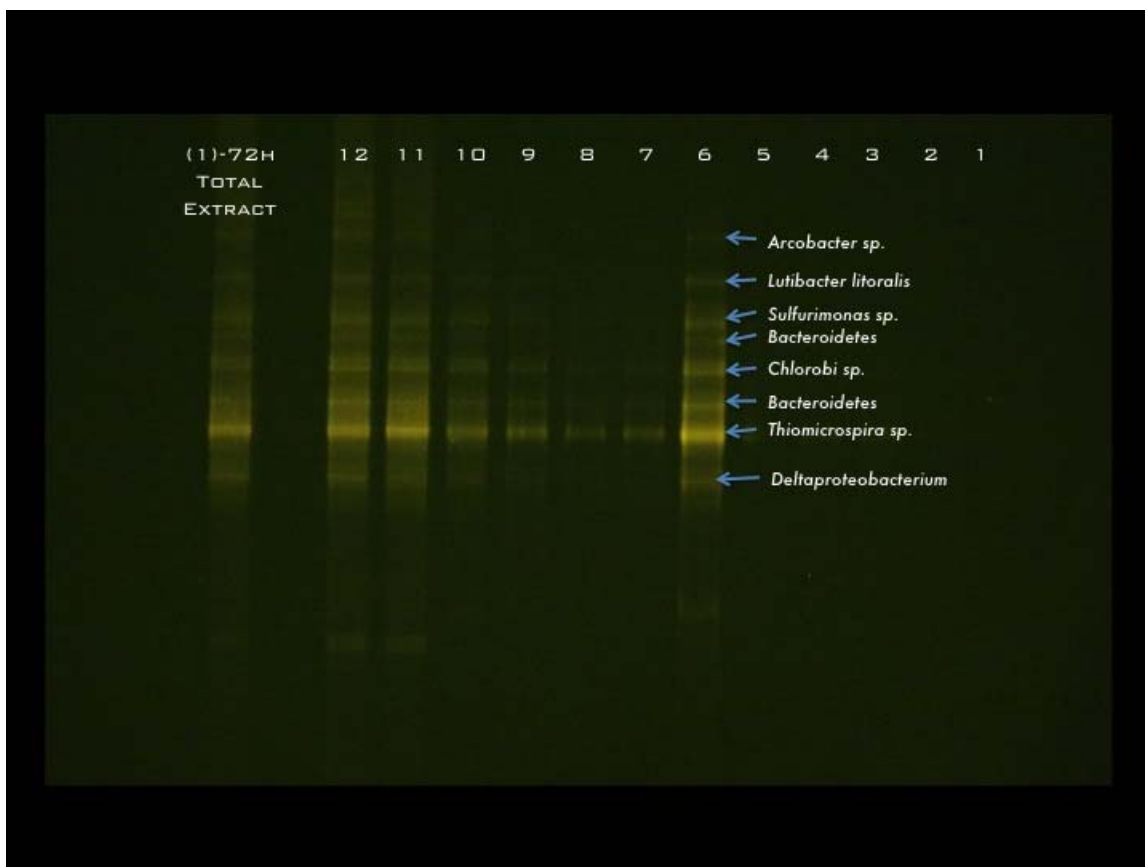




**Figure 7: Sequenced Epsilonproteobacterial DGGE bands.** Primer pair E1f/E2r was used with a touchdown DGGE program described above. Sequences were BLASTed and labels are the nearest classified sequence from the BLAST search  $\geq 95\%$  identity for  $\geq 90\%$  of the sequence.

#### ***DGGE 16S rRNA Sequencing:***

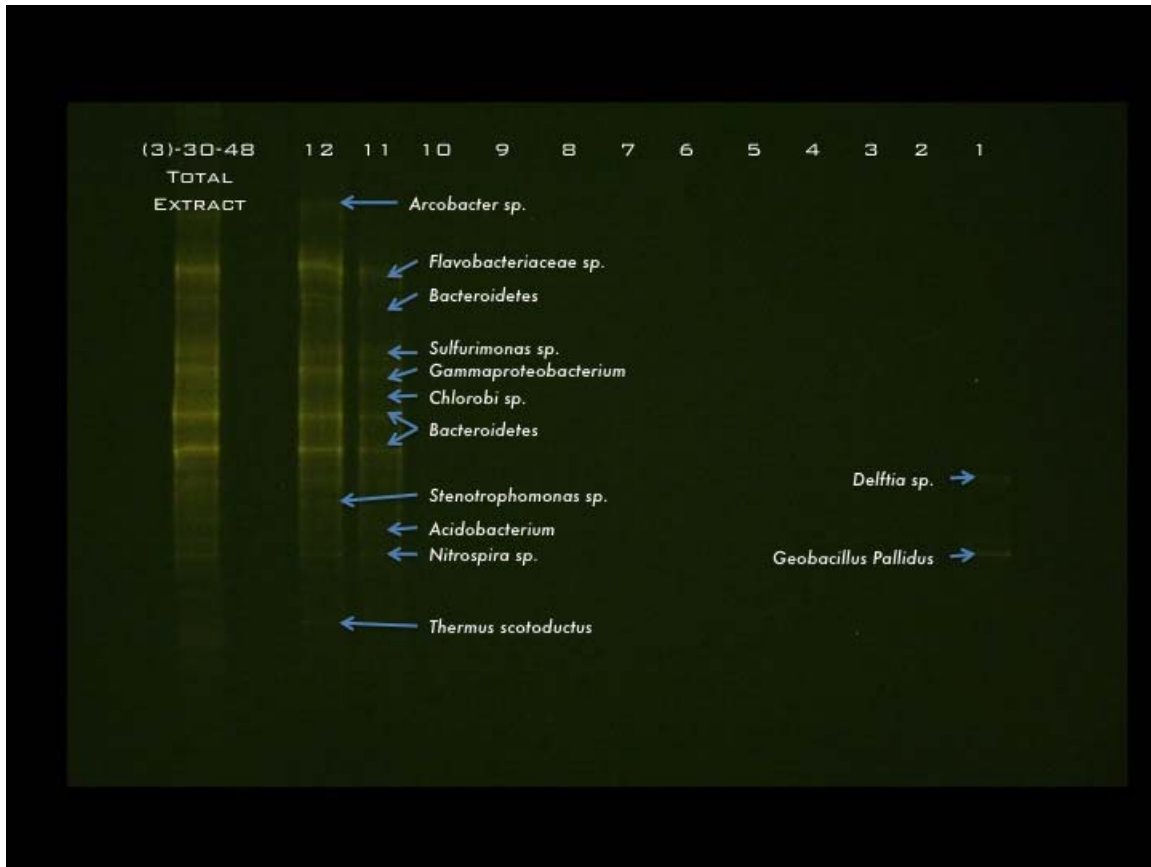
DGGE gels were made with GC-clamped PCR products from every fraction of all SIP separated incubations: (1)-72, (3)-30-48, (3)-50-48, (5)-30-48, (5)-50-48 (Figures 8-12 respectively). Major bands were excised for sequencing. The bands were labeled on each DGGE image by the nearest classified sequence (as determined by the BLAST program) to the actual band sequence.



**Figure 8: (1)-72 SIP DGGE gel.** Primer pairs GC-341f/907r were used to generate the 16S rDNA PCR products. Fraction 6 represents the “heaviest” DNA that was quantifiable with PicoGreen Assay and fraction 12 represents the “lightest” DNA. Labeled bands represent quality sequences with >200 bases of 16S rRNA information. Total Extract is a lane made from PCR products of the (1)-72 incubation DNA before it was separated by SIP.

All the bands present in the heavily labeled lane (lane 6) appear to be present in the unlabeled lanes (11-12), and no distinctive pattern of lanes shifting between the “heavy” and “light” can be seen. This may indicate that 72 hours was beyond the point at which labeled chemoautotrophic organisms began to die, spilling their labeled molecules into the larger incubation pool. The *Arcobacter sp.* and *Sulfurimonas sp.* bands in these lanes are very faint in comparison to the bands found in Figure 7 using Epsilonproteobacterial specific primers. Band intensity in all of these DGGE images does not represent quantity of DNA or cells present in the incubation because the PCR used to create the products was non-quantitative (Ahn et al., 2009). This means that although *Thiomicrospira sp.* appears to be the brightest band on the gel, it does not mean that it is the predominant organism in the incubation (Gammaproteobacteria account for only 2% of (1)-72 CARD-FISH cell

counts). *Arcobacter sp.* and *Sulfurimonas sp.* represent the only two Epsilonproteobacteria in this gel.

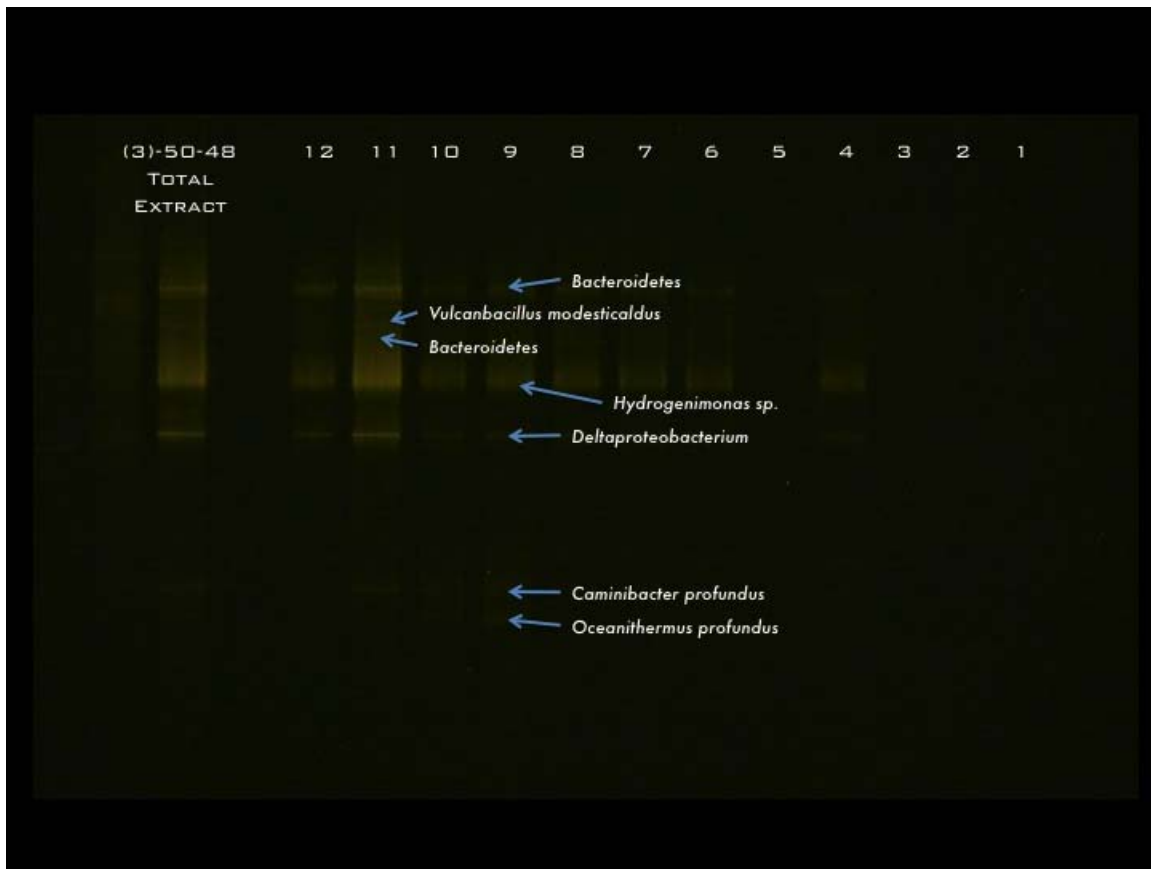


**Figure 9: (3)-30-48 SIP DGGE gel.** Primer pairs GC-341f/907r were used to generate the 16S rDNA PCR products. Fraction 10 represents the “heaviest” DNA that was quantifiable with PicoGreen Assay and fraction 12 represents the “lightest” DNA. PicoGreen Assay could not quantify any DNA in fraction 1 (which could represent a contamination). Labeled bands represent quality sequences with >200 bases of 16S rRNA information. Total Extract is a lane made from PCR products of the (3)-30-48 incubation DNA before it was separated by SIP.

This separation appears to be the least pronounced of the SIPs. Only lanes 12 and 11 (excluding lane 1 due to it being a probable contamination) showed bands capable of being sequenced, and there does not appear to be any pattern in the banding from 12 to 11.

Both (1)-72 and (3)-30-48 DGGEs contained bands of *Chlorobi sp.* green sulfur bacteria (the sequences in the two DGGEs are not identical). Similar to the (1)-72 gel the *Arcobacter sp.* and *Sulfurimonas sp.* bands are faint relative to other bands.

*Arcobacter* sp. and *Sulfurimonas* sp. represent the only two Epsilonproteobacteria in this gel.



**Figure 10: (3)-50-48 SIP DGGE gel.** Primer pairs GC-341f/907r were used to generate the 16S rDNA PCR products. Fraction 8 represents the “heaviest” DNA that was quantifiable with PicoGreen Assay and fraction 12 represents the “lightest” DNA. Labeled bands represent quality sequences with >200 bases of 16S rRNA information. Total Extract is a lane made from PCR products of the (3)-50-48 incubation DNA before it was separated by SIP.

The (3)-50-48 SIP DGGE shows some banding patterns between the fractions. *Oceanithermus profundus* band appear only in lanes 9 and 10, the *Vulcanbacillus modesticaldus* (a heterotrophic bacteria) band appears clearly visible only in lane 11, and *Hydrogenimonas* sp. is a constant band from the “light side” of the gel (lane 12) to the “heavy side” of the gel (lane 6; also present in lane 4). *Vulcanbacillus modesticaldus*, *Hydrogenimonas* sp., *Caminibacter profundus*, and *Oceanithermus profundus*, all represent thermophilic species with optimum growth temperatures at around 55°C. *Hydrogenimonas* sp. and *Caminibacter profundus* are the two Epsilonproteobacterial sequences in this gel.



**Figure 11: (5)-30-48 SIP DGGE gel.** Primer pairs GC-341f/907r were used to generate the 16S rDNA PCR products. Fraction 9 represents the “heaviest” DNA that was quantifiable with PicoGreen Assay and fraction 12 represents the “lightest” DNA. Labeled bands represent quality sequences with >200 bases of 16S rRNA information. Total Extract is a lane made from PCR products of the (5)-30-48 incubation DNA before it was separated by SIP.

There is no definite banding pattern in the (5)-30-48 gel (with the exception of the *Delftia sp.* band which is most likely a contaminant). The *Arcobacter sp.* band, which is present in all 30°C incubation DGGE gels, is much brighter in this gel than in previous ones. *Arcobacter sp.* is the only Epsilonproteobacteria present in this gel.

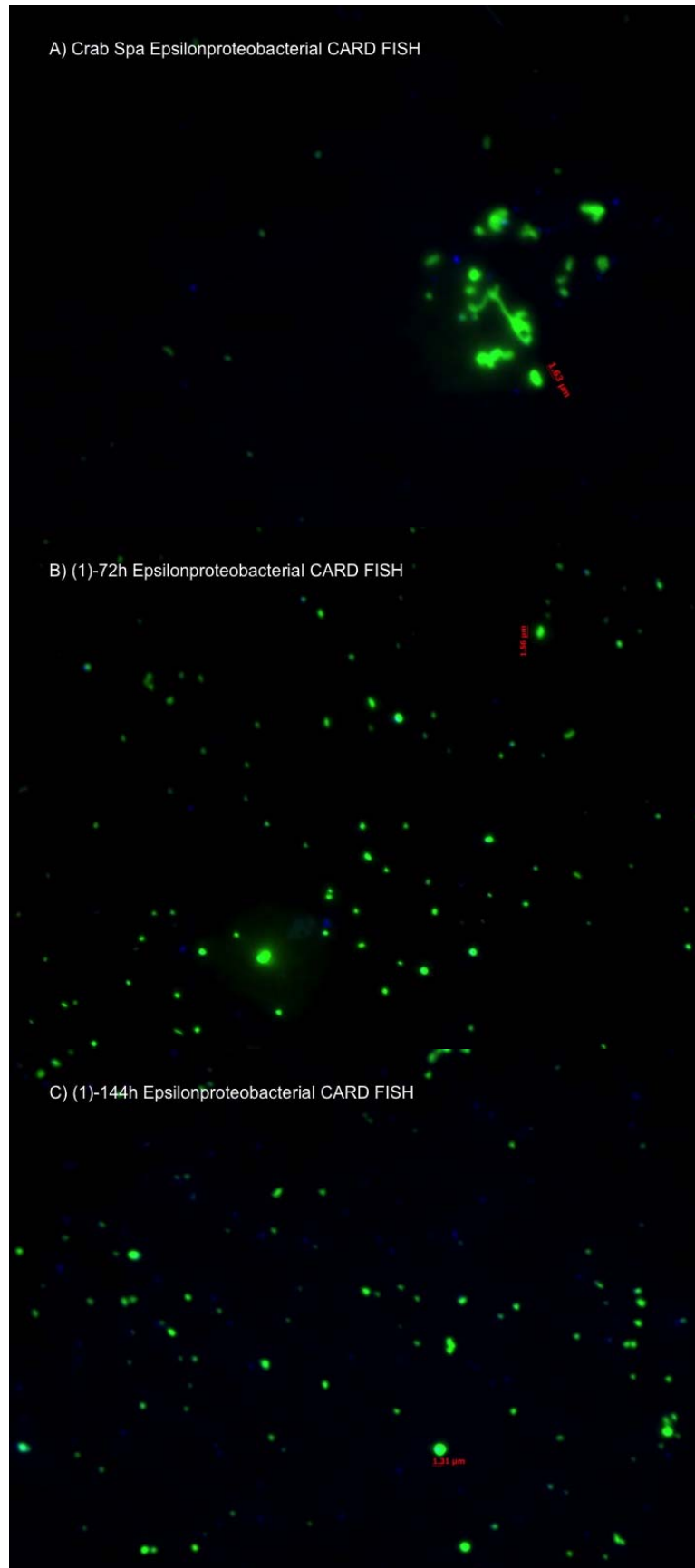


**Figure12: (5)-50-48 SIP DGGE gel.** Primer pairs GC-341f/907r were used to generate the 16S rDNA PCR products. Fraction 9 represents the “heaviest” DNA that was quantifiable with PicoGreen Assay and fraction 12 represents the “lightest” DNA. Labeled bands represent quality sequences with >200 bases of 16S rRNA information. Total Extract is a lane made from PCR products of the (5)-50-48 incubation DNA before it was separated by SIP.

*Caminibacter profundus*, the only Epsilonproteobacterial sequence in the (5)-50-48 gel, displays a banding pattern that peaks in lane 9 (the “heaviest” fraction quantifiable with PicoGreen assay). This is evidence of a chemoautotrophic organism becoming labeled over the course of the incubation. *Desulfovibrionales* is a subdivision of Deltaproteobacteria classified as sulfate-reducing bacteria (SRBs). *Marinitoga* is a genus of thermophilic bacteria normally associated with hydrothermal vent sites (Postec et al., 2005, 2007).

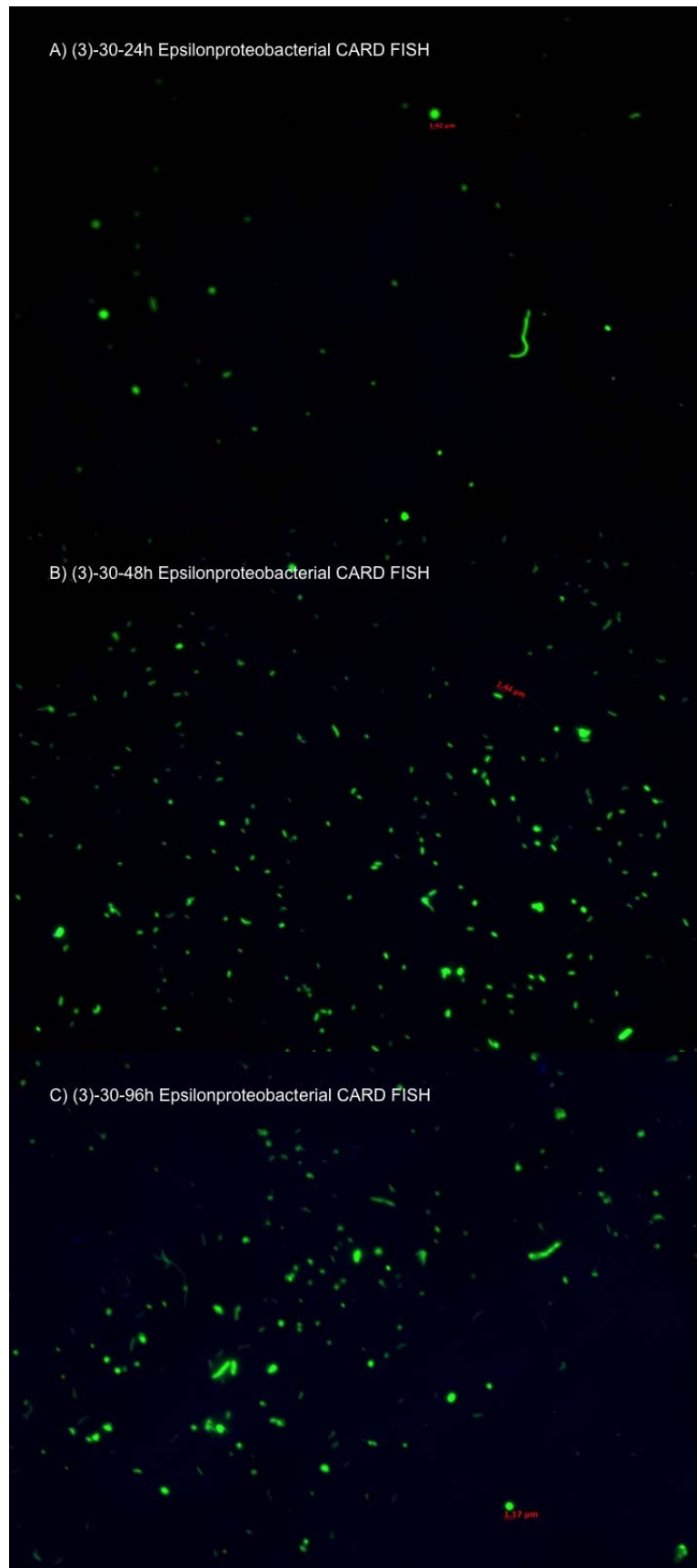
***CARD-FISH Microscopy:***

Images were taken using the 40x objective lens and overlaid to produce composite images of DAPI and A<sub>488</sub> fluorescence. The progression from 24 hours to 96 hours shows changes in the morphologies, cell quantities, and overall community over time (Figures 13-18).

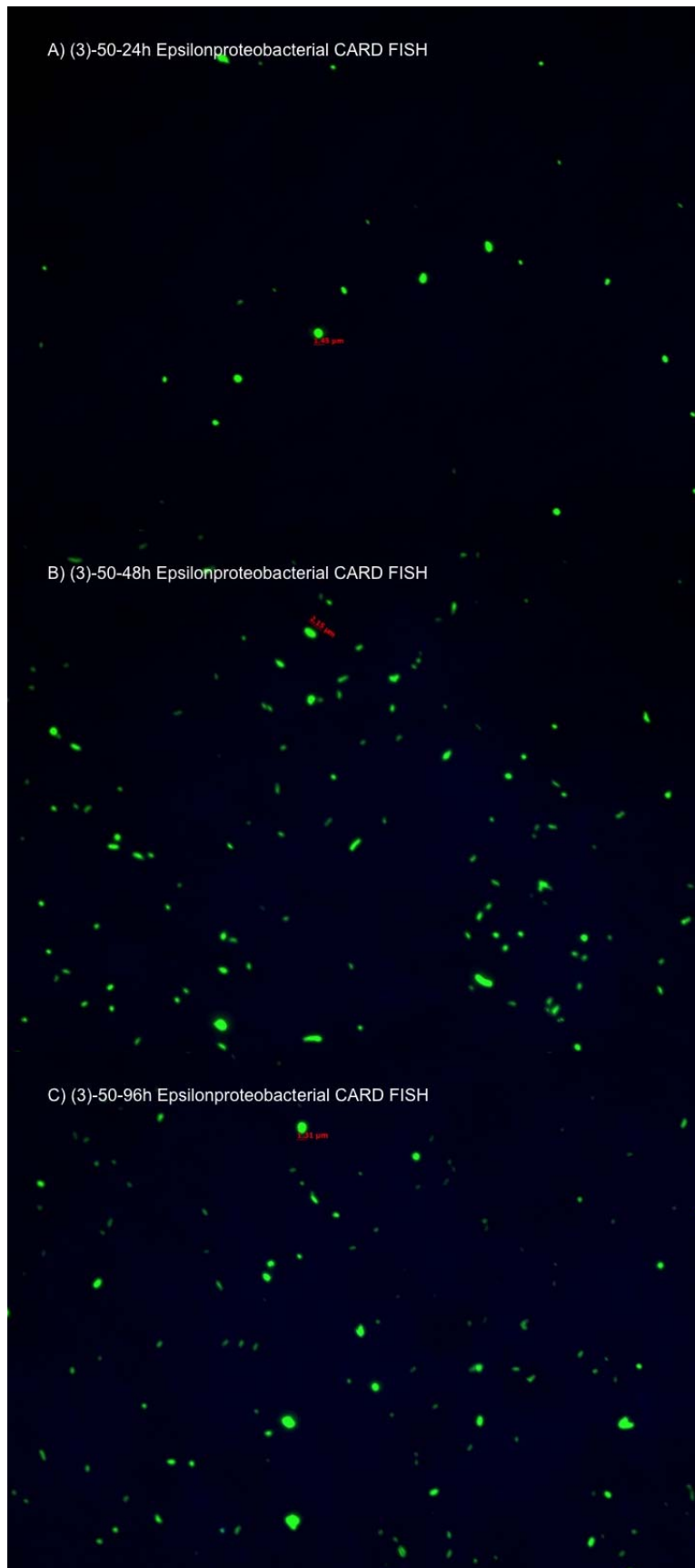


**Figure 13: Epsilonproteobacteria CARD-FISH incubation (1).** Green cells are Epsilonproteobacterial cells (DAPI + A<sub>488</sub>) and Blue cells are non-Epsilonproteobacterial cells (DAPI without A<sub>488</sub>).

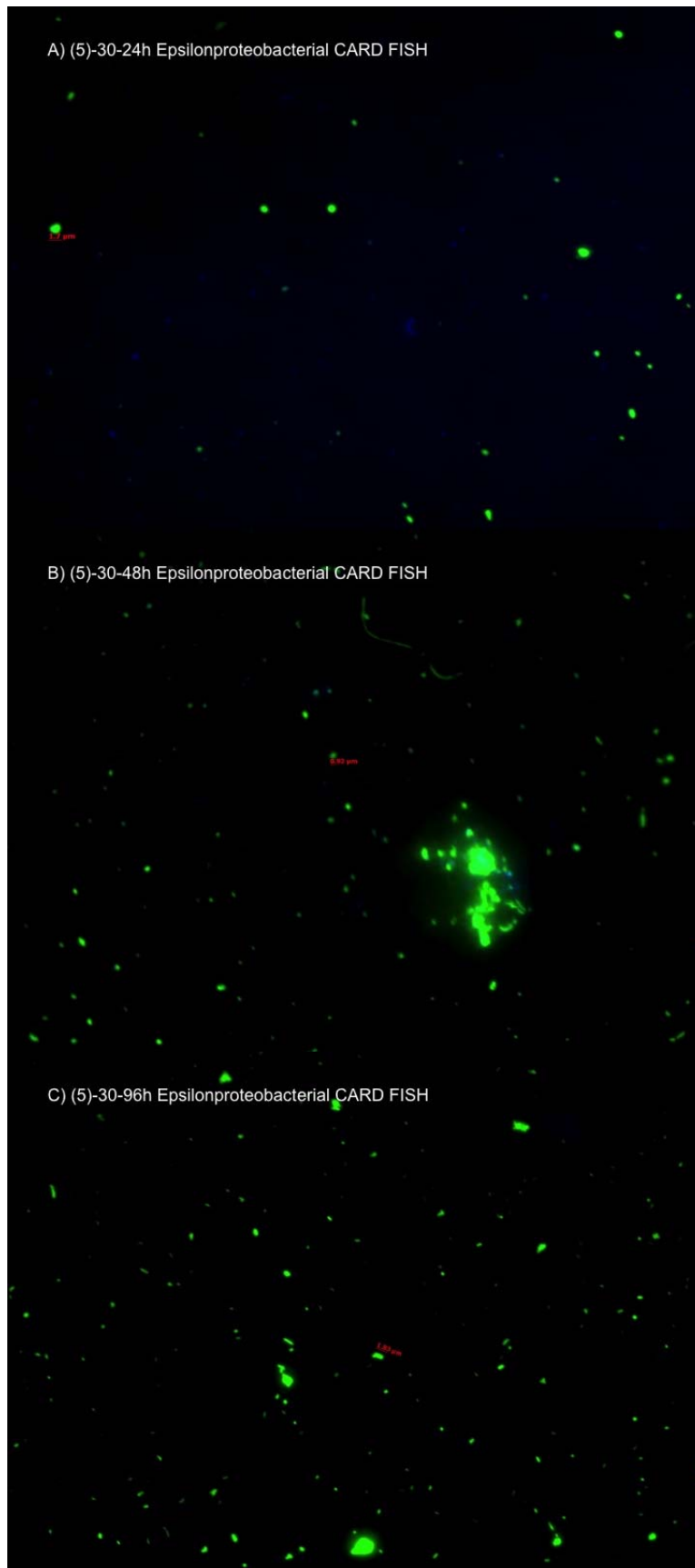




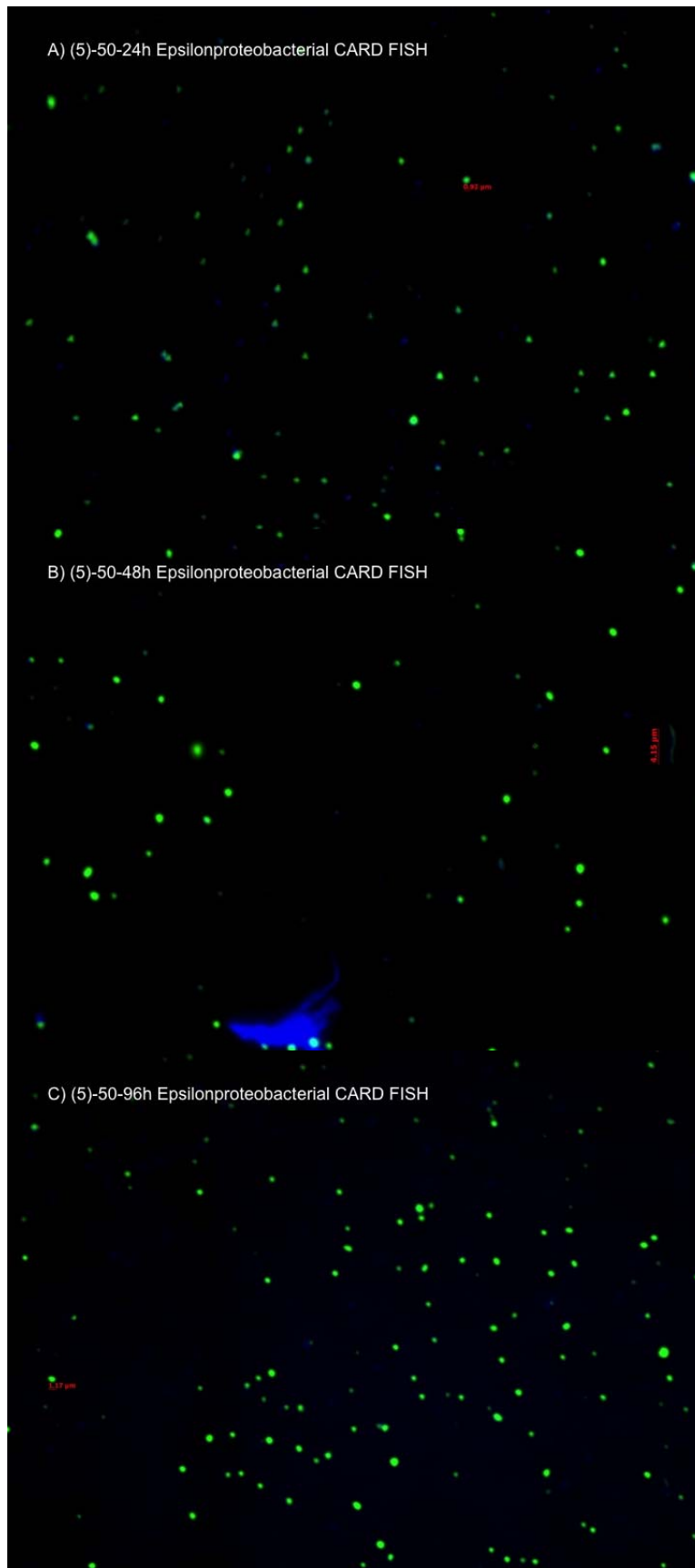
**Figure 14: Epsilonproteobacteria CARD-FISH incubation (3)-30.** Green cells are Epsilonproteobacterial cells (DAPI + A<sub>488</sub>) and Blue cells are non-Epsilonproteobacterial cells (DAPI without A<sub>488</sub>).



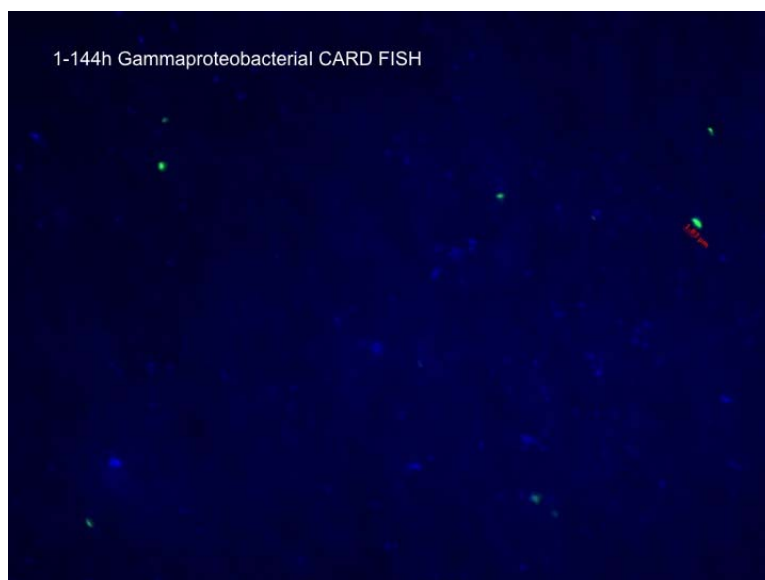
**Figure 15: Epsilonproteobacteria CARD-FISH incubation (3)-50.** Green cells are Epsilonproteobacterial cells (DAPI + A<sub>488</sub>) and Blue cells are non-Epsilonproteobacterial cells (DAPI without A<sub>488</sub>).



**Figure 16: Epsilonproteobacteria CARD-FISH incubation (5)-30.** Green cells are Epsilonproteobacterial cells (DAPI + A<sub>488</sub>) and Blue cells are non-Epsilonproteobacterial cells (DAPI without A<sub>488</sub>).



**Figure 17: Epsilonproteobacteria CARD-FISH incubation (5)-50.** Green cells are Epsilonproteobacterial cells (DAPI + A<sub>488</sub>) and Blue cells are non-Epsilonproteobacterial cells (DAPI without A<sub>488</sub>).

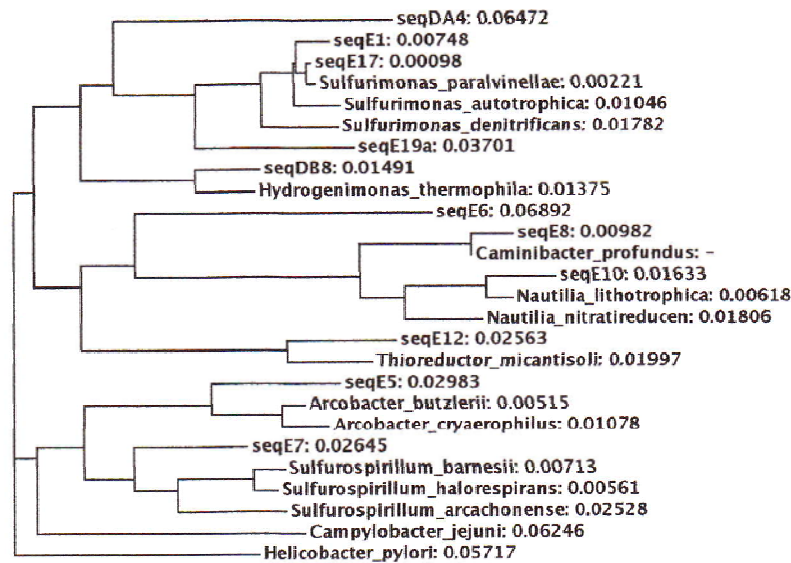


**Figure 18: Gammaproteobacterial CARD-FISH incubation (1)-144.** Green cells are Gammaproteobacterial cells (DAPI + A<sub>488</sub>) and Blue cells are non-Gammaproteobacterial cells (DAPI without A<sub>488</sub>). (1)-144 had the largest percentage of Gammaproteobacteria of any incubation (6.0%).

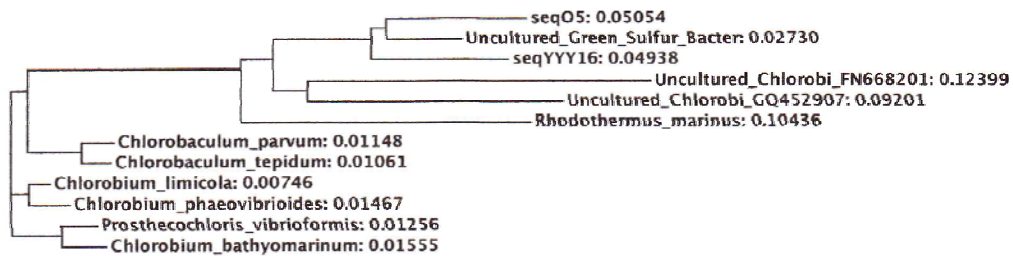
The images for (3)-30-48 and (3)-50-48 show the most obvious changes in morphology over time. Mostly coccoid cells on these 24 hour filters change to a variety of large rods, small rods, and elliptical shaped cells at 96 hours. Clusters of cells similar to those in Figure 16 Plate B and Figure 17 Plate B were much more common in the 48 hour samples of all conditions than at any other time point.

### ***Phylogenetic Trees:***

Two phylogenetic trees were constructed to show the relation between actual DGGE band sequences and the nearest classified sequences (as determined by BLAST). Figure 19 shows the major Epsilonproteobacterial sequences from this study and Figure 20 shows the green sulfur bacterial sequences.



**Figure 19: Epsilonproteobacterial Phylogram.** Branch lengths are proportional to the amount of inferred evolutionary change and the pairwise distances follow the sequence names.



**Figure 20: Green Sulfur Bacteria Phylogram.** Branch lengths are proportional to the amount of inferred evolutionary change and the pairwise distances follow the sequence names.

## Discussion:

The major conclusion of this research is that “Epsilonproteobacteria” are the dominant microbial players in the diffuse flow fluid. The CARD-FISH data shows their numbers at around 70% initially when the fluid is first collected and that percentage growing within the first 24 hours in every single incubation condition. Epsilonproteobacteria specific primers (E1f/E2r) were able to amplify out all of the sequences (in addition to some extras) of epsilon microbes found in the SIP separation DGGEs. The filamentous sulfur observed in all 30°C incubations, both microaerophilic and anoxic, was indicative at the time of the presence of *Arcobacter*

*sp.* (Wirsén et al., 2002). The sequence data show that this was indeed the case and lends itself to the possibility that an *Arcobacter* species could be capable of growth under anoxic conditions. The presence of *Hydrogenimonas sp.* in incubation condition (3) not containing a hydrogen headspace is surprising (Takai et al., 2004). However concurrent data gathered with gastight samplers in the Seewald Lab onboard the AT15-38 *Atlantis* cruise showed the level of H<sub>2</sub> gas in the initial fluid emanating from Crab Spa to be 3 μM.

The counterintuitive nature of their seeming to be higher Epsilonproteobacterial cell counts than bacterial cell counts can be explained by the particulars of the CARD-FISH process: some cells disintegrate during the permeabilization or hybridization processes especially with higher concentrations of formamide (Epsilonproteobacterial = 55% formamide, Bacterial = 35% formamide) and therefore are not counted properly if they no longer exist intact on the filter (Furukawa et al., 2006). Therefore CARD-FISH in complex microbial communities sometimes produces skewed results, which cannot be corrected because the nature of the disintegrating microorganisms is unknown. The trends observed by CARD-FISH over time are not subject to these affects since theoretically disintegrating cells would disintegrate in all filters treated under the same conditions equally. An overall decrease in Epsilonproteobacteria and increase in Gammaproteobacteria lead to the conclusion that community succession was an important biological process within the incubations.

The observational data also argues for community succession over time. The complete disappearance of flocculent filamentous sulfur after 72 hours and the appearance of yellow precipitates after 144 hours (in condition 1) argue for the chemical byproducts of one group of organisms creating a new resource for the chemical processes of a second group. Research using in situ growth chambers which accidentally acquired temperature gradients during incubation showed the same rapid succession amongst vent microorganisms (Reysenbach et al., 2000). GeoChip-based analysis of vents microorganisms at the Juan de Fuca Ridge were equally conclusive about the “rapid dynamic succession” in response to changing vent conditions over time (Wang et al., 2009).

Rapid succession would seriously affect one of the crucial factors in DNA SIP experimentation: the length of incubation with  $^{13}\text{C}$  label. Too little time and the cellular DNA will not be adequately labeled to perform a clean separation. Too much time and the experimenter runs the risk of cellular turnover producing a cross feeding problem which could label all populations. There is ample evidence that cross-feeding may have played a significant role in the incubations outlined above. All 5 SIP separations showed evidence that *Bacteroidetes* populations had bands present in fractions containing the heaviest DNA, and there are no known *Bacteroidetes* capable of chemoautotrophy. *Fimicute* and *Actinobacteria* sequences found in heavy fractions also lend credibility to the theory that the incubation period was in excess of what was needed to label only chemoautotrophs.

Experimental bacteria DB8 (closely related to *Hydrogenimonas* sp., Figure 19) is likely, based on previous experiments (Takai et al., 2004), to have a doubling time of approximately 70 minutes. Bacteria E8 (closely related to *Caminibacter* sp., Figure 19) could have a doubling time close to 90 minutes (Alain et al., 2002), and bacteria E10 (closely related to *Nautillia lithitrophica*) could have a doubling time of on the order of 4 hours (Miroshnichenko et al., 2002). In an experimental environment of 30%  $^{13}\text{C}$  bicarbonate ion approximately 6-7 generations would be necessary to adequately label DNA, and for all three of the microorganisms listed above that would require a total incubation time of 24 hours or less. Since visible changes in the incubations were noticeable at 12 hours it's reasonable to conclude that future experiments using SIP on vent fluids should contain time points within the first 24 hours.

Recent reviews and studies using DNA Stable Isotope Probing have emphasized “experimental timing” as a critical aspect of successful DNA labeling without cross feeding (Chen and Murrell, 2010, Webster et al., 2010). They also point out that a critical error in this experiment was the lack of an unlabeled control incubation. Despite the fact that hydrothermal fluid of this nature is extremely hard to procure in great quantities, a control incubation could be set up in future iterations of this experiment containing no  $^{13}\text{C}$  label. This would allow for the examination of a DGGE with no “heavy” DNA. This control would also address another issue pointed out by



Buckley et al. (2007); microorganisms have a widely variable G+C content in their DNA and  $^{13}\text{C}$  SIP is sometimes biased by DNA with >60% G+C content separating out in heavier fractions and presenting the illusion of being labeled DNA.

Despite the difficulties encountered with this DNA SIP experiment it is by no means a technique that should be abandoned for hydrothermal vent research. The data shows that there was plenty of extracted DNA to perform the procedure several times over, and that separation as evidenced by density results (Figure 4) and fraction quantification (Figure 5) definitely occurred. The  $\delta^{13}\text{C}$  data indicates that labeling of DNA was beyond the necessary threshold of  $\sim 5\mu\text{mol } ^{13}\text{C}/\text{ml}$  fluid (Chen and Murrell, 2010). This leaves only the timing of the experiment to eliminate cross feeding as the critical factor when designing a second generation of experiments for hydrothermal vent SIP. Highly successful DNA SIP experiments have involved targets which were very specific within their community structure; the search for sulfate reducing toluene degraders for example (Winderl et al., 2010). The search for chemoautotrophs in a pool of mixotrophs, heterotrophs and possibly photoautotrophs is a more complex task complicated by the fact that sampling is a “one-shot” deal.

The possibility of hydrothermal vent photoautotrophy in this experiment is evidenced by the two Chlorobi-like sequences found in the DGGE gels of condition (1)-72 and (3)-30-48 (sequences 05 and YYY16 from Figure 20). In 2002 White and her colleagues postulated and found black body radiation associated with high temperature hydrothermal venting. They called this phenomena geothermal light, and classified it as the “thermal radiation due to the high temperature of hydrothermal fluid,” however they ended their remarks with the statement that no obligate microorganism could harness this very low intensity light. That was shown to be incorrect when Beatty et al. (2005) characterized *Chlorobium bathyomarinum*, an anaerobic obligate photoautotrophic green sulfur bacteria, extracted from the hydrothermal vent system at EPR 9°N. The present study has found two 16S rDNA sequences which have phylogenetic relatives to green sulfur bacteria but are not closely related to the original discovery of *Chlorobium bathyomarinum* (Figure 20). Sequence 05 was found in the (1)-72 microaerophilic incubation and sequence

YYY16 in the (3)-30-48 anaerobic incubation. It is important to note that although the water baths containing all of the incubations were partially covered to prevent runaway evaporation, that covering was only partial and fluorescent light (which was “on” in that portion of the lab 24 hours a day) could penetrate the incubation bottles at low levels. *Chlorobium bathyomarinum* was found to be resistant to brief aerobic conditions, which helps to understand why a Chlorobi-like organism might thrive in the microaerophilic environment of condition 1. Depending on the growth rates of these organisms it is possible, although the data only suggests this, that these green sulfur bacteria were growing on the  $^{13}\text{C}$  label and increasing the mass of their DNA not through chemoautotrophy, but through photoautotrophy.

There were other unexpected sequences parsed out from DNA SIP DGGE gel bands. *Lutibacter litoralis* (found in condition (5)-30-48) is classified as an aerobic heterotroph (condition 5 was designed to be anaerobic) with the incubation temperature of 30°C being the maximum of its growth tolerances (Choi and Cho, 2006). *Caldithrix* is a genus of strictly anaerobic mixotrophic thermophiles which grow optimally at 60°C (Miroshnichenko and Bonch-Osmolovskaya, 2006). Finding this organism growing and apparently labeled in a 30°C incubation would not necessarily be expected. *Thermus scotoductus* at the base of the (3)-30-48 gel in lane 12 (Figure 9) is an organism originally isolated from African gold mines capable of growing on nitrate (nitrate was added to all condition 3 and 5 incubations),  $\text{Fe}^{3+}$ ,  $\text{Mn}^{4+}$ , or Sulfur as terminal electron acceptors and capable of reducing  $\text{Cr}^{4+}$ ,  $\text{U}^{4+}$ , and  $\text{Co}^{3+}$  (Balkwill et al., 2004). Above the *Thermus scotoductus* band is *Nitrospira*, which is a genus of nitrite-oxidizing bacteria. A sequence for *Thioreductor micantisoli* (an Epsilonproteobacteria previously described as mesophilic with temperature maximums of 42°C) was found after 96 hours of incubation with hydrogen at 50°C (Figure 7) (Nakagawa et al., 2005). *Delftia* sp. and *Geobacillus pallidus* were found once during this study in a negative control lane (data not shown), and are therefore believed to be contaminating sequences.

*Geobacillus pallidus* is a strict aerobe with growth temperatures in excess of 30°C (it was sequenced from the (3)-30-48 gel, an anaerobic incubation) and was originally isolated from raw sewage (Zhou et al., 2008). *Delftia* is a genus of

organisms which cannot survive in the marine environment because of the high levels of NaCl ions (Wen et al., 1999). *Delftia* were associated with the widely publicized bacterial contamination of contact lens solutions (Willcox et al., 2010) and are also found in pasteurized milk, ice machines, and inside human aneurysms. Therefore it is highly unlikely that these organisms exist in hydrothermal fluid. It is much more likely that they were laboratory contaminants either from the incubations on the ship or from DNA manipulation in the lab at Woods Hole.

The presence of potentially labeled *Lutibacter litoralis* in an anaerobic incubations presents the question of whether there was molecular oxygen present in (5)-30-48. *Lutibacter* was also found in (1)-72 but that condition was microaerophilic and contained oxygen from the start of the incubation. The three major biological pathways of O<sub>2</sub> production are from photosynthesis, chlorate respiration, and detoxification of reactive oxygen species, none of which are likely to have occurred in any of the incubations. In March of 2010 a fourth way of producing molecular oxygen through biological pathways was published by Ettwig et al., and it involves oxygenic bacteria involved in the nitrite-driven anaerobic oxidation of methane. In this new pathway nitric oxide (NO) is converted by an as yet unknown enzyme(s) to dinitrogen and molecular oxygen. The bacteria responsible for this process, *Methyloirabialis oxyfera*, was discovered by metagenomic analysis of genomes from ditch sediments. There is no evidence of this bacteria or any like it existing in hydrothermal fluid, but the presence of 12μM methane in Crab Spa fluid (Seawald Lab gastight samplers), and the addition of 1mM Nitrate (easily converted to Nitrite) does make the existence of an oxygenic microorganism in hydrothermal fluid plausible.

The apparent “cross-feeding” <sup>13</sup>C labeling of so many cells in the incubations presents the possibility of one or more predatory bacteria in the hydrothermal fluid. Examination of over 100 CARD-FISH filters gave no indication of Protist grazers within the incubation, but that does not rule out the possibility that bacterial grazers were present. Predatory bacteria species have been classified from *Proteobacteria* ( $\alpha$ -,  $\beta$ -,  $\gamma$ -), *Chloroflexi*, *Bacteroidetes*, *Actinobacteria*, and most recently *Flavobacteria* (Banning et al., 2010). All of these bacterial groups (exception of Chloroflexi) were

found in the sequence space of this experiment. Sequences of *Bacteroidetes* and *Flavobacterial* organisms from this research were handed off to Erin Banning, the author of the 2010 predatory bacteria paper, and his analysis showed that at least three sequences (seqT2, seqP1, seqDB5, data not shown) were somewhat related to those in his study which were proven to be predatory. The existence of predatory bacteria within the incubations would help explain why most all microbial populations appear to be labeled with  $^{13}\text{C}$  after 48 hours. These bacteria could potentially lyse chemoautotrophic cells, spilling their  $^{13}\text{C}$  labeled contents into the media for heterotrophic consumption. The fall in cell counts from 48 to 96 hours in condition (5)-50 is also possible evidence for the existence of predatory bacteria. A SIP experiment performed with the predatory bacteria *Lysobacter* showed that after feeding  $^{13}\text{C}$  labeled *E. coli* to the predators, labeled *Lysobacter* RNA was detectable after only one hour of incubation (Lueders et al., 2006).

Choice of PCR primers is important to any experiment relying on DGGE for isolation of specific organisms. In this experiment the primer pair 341f/907r, a widely used primer “universal” to bacteria, was used for DNA SIP DGGE gels. However the results of those gels do not reflect the numbers of Epsilonproteobacteria counted with CARD-FISH. This could possibly be due to differential amplification, a problem researched by Sipos et al. in 2007. Their experiments showed that even a single mismatch in the primer pair used could have an effect that would multiply through the reaction preferentially amplifying minority sequences over majority ones. Using probeMATCH (online DNA probe resource) it was possible to calculate the percentage of known sequences that would bind exactly to the precise sequence of 341f: 67% of known Epsilonproteobacteria, 73% of known Proteobacteria, and 77% of known Bacteroidetes. The Sipos research also showed that annealing temperatures can be lowered to the lowest state that excludes non-specific binding and that will greatly decrease the effect of differential amplification (they found that cycle number played no part in differential amplification). One of their concluding thoughts, which applies specifically to this research, was that touchdown PCR programs are detrimental to representative amplification because they do not follow the rule established by their research of lowest annealing temperature possible. It is highly

likely that the apparent lack of strong banding from Epsilonproteobacterial sequences and the apparent strength of the Bacteroidetes bands is due to this PCR bias. If Bacteroidetes FISH probes were used to count the actual numbers of these bacteria within the incubation conditions it would likely lend more evidence to this theory.

### ***Conclusion:***

Epsilonproteobacteria (*Arcobacter*, *Caminibacter*, *Hydrogenimonas*, *Sulfurimonas*, *Sulfurospirillum*, *Nautilia*, and some unclassified geneses) dominate the initial stages of diffuse flow vent incubations in cell number and probably chemoautotrophically. DNA Stable Isotope Probing is an investigative method that can be successfully used on hydrothermal vent samples with the caveat that timing of the experiments is critical, and the possibility of predatory bacteria must be taken into consideration. Further research needs to be done on the green sulfur bacteria found at EPR 9° North; the potential implications for the evolution of photosynthetic life on the planet are enormous. DGGEs for experiments such as this one should be performed with more than one set of PCR primers in order to ensure that the sequence space is being appropriately parsed, and a touchdown PCR program should not be used. The diffuse flow hydrothermal vent fluid is a very complex community of organisms which can adapt quickly to changing conditions and turnover in rapid succession on the order of hours and not days. Future experiments with this fluid should be conducted on a shorter time scale.

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